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SOME ASPECTS OF THE MICROBIOLOGY OF CHEESE RIPENING INVESTIGATED USING ASEPTIC MANUFACTURING TECHNIQUES

A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Food Technology at Massey University

Keith Willis Turner

August 1988

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ABSTRACT

The effect of non-starter lactic acid bacteria (NSLAB) on the texture and flavour development in cheese was investigated using Cheddar cheese matured in impermeable cheese barrier bags. Cheeses made normally in open vats (and contaminated with adventitious NSLAB) were compared with NSLAB-free cheeses.

To produce NSLAB-free cheeses, two totally enclosed cheese-making vats were designed and constructed. These vats were sterilized by soaking overnight with Iodophor, then steam flushed for 1 hour. This procedure was sufficient to exclude NSLAB but did not produce complete sterility as evidenced by the growth of bacillus-type organisms in UHT-treated milk incubated in the sterilized vats.

The thermal death characteristics of representative strains of NSLAB showed that most species would not survive milk pasteurization temperatures. However, two species, Lactobacillus casei var casei and Leuconostoc lactis were sufficiently resistant that, if present as the dominant flora of a raw milk, they could survive into the curd. Therefore, for aseptically manufactured cheeses, the milk was obtained by careful milking of the cows, to avoid NSLAB contamination. In the pasteurized vat-milks, the total bacterial count was routinely less than 1 cfu/mL.

A panel of tasters was trained to determine the texture and flavour of the cheeses using attribute scaling techniques. Six textural and 5 flavour attributes were defined, and the intensities of these were monitored in the cheeses at various ages up to 9 months.

The aseptic cheese-making procedure effectively eliminated NSLAB contamination from the cheeses. However, when cheeses in bags were matured for long periods, a surface flora of adventitious NSLAB developed, apparently by contamination during sampling. Despite all sampling precautions, the only method found to satisfactorily prevent this contamination was waxing of the cheese surfaces prior to bagging. It was also observed that in adventitiously contaminated cheeses, the count of NSLAB in the surface 1-2 mm was at least 10 times the count in the remainder of the cheese.

The texture and flavour of Cheddar cheeses made without NSLAB and matured at 10 C for 9 months could not be distinguished from cheeses with adventitious NSLAB matured under the same conditions. In equivalent cheeses matured at 15C, the textures were again identical but the cheeses with NSLAB had greater intensities of sharpness and sulphide than the cheeses without NSLAB. Thus, starter alone appears to be the predominant contributor of those compounds which produce Cheddar cheese flavour and NSLAB, although present, do not normally contribute to flavour production.

In a final part, the growth and citrate utilizing capabilities of three leuconostoc organisms were determined in a washed-curd cheeses. Again, NSLAB-free cheeses were made since there are

currently no effective means of differentiating between leuconostoc and NSLAB organisms, and NSLAB organisms can also utilize citrate. While all three strains were capable of rapid growth to levels around 10^7 cfu/mL in RSM, only one grew significantly in the cheeses. This strain was capable of fermenting citrate in the absence of a carbohydrate energy source and removed the citrate present in brine-salted cheeses within 1 month at either 10 C or 15 C.

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Literature Review and General Introduction

Current Theories Of Flavour Development in Cheese

The mechanism of flavour development in cheese (and especially Cheddar cheese) has been extensively researched over the last 80 years and many reviews have been published on the subject. Indeed it has been suggested that "...the number of reviews appears to be inversely proportional to the progress in understanding it" (Reiter and Sharpe, 1971). Rather than exhaustively reviewing the total literature on cheese flavour development, this review seeks to place in context the research project undertaken, the aim of which was to further elucidate the role of microorganisms, particularly non-starter lactic acid bacteria (NSLAB), in flavour development.

Since by far the most significant cheese to the New Zealand Dairy industry is Cheddar cheese, discussion will be restricted mainly to that cheese. Cheddar cheese provides a model for understanding the general principles of cheese flavour development, since the flavour is not modified by a deliberately added secondary organism (blue or white moulds, smear organisms or propionibacteria). Interestingly it has also been studied more extensively than any other cheese variety.

While many of the general principles have been established, there is continuing speculation as to what compounds contribute to cheese flavour and what conditions are required for their formation in cheese. Cheese flavour research can be broadly divided into two categories:

i) The isolation and identification of the chemical compounds in the cheese which may contribute to the perceived flavour (for reviews see for example Marth, 1963; Forss and Patton, 1966; Schormuller, 1968; McGuigan, 1975; Behnke, 1980; Aston and Dulley, 1982; and Hemme _et al_., 1982);

and ii) The investigation of those factors (e.g. chemical and bacterial composition) which influence or control flavour development (for reviews see for example Mabbitt, 1961; Reiter _et al_., 1966; Jacquet and Lenoir, 1968; Adda _et al_., 1982; Grappin _et al_., 1985; Rank _et al_., 1985).

Some reviewers (see for example Fryer, 1969; Reiter and Sharpe, 1971; Evans and Mabbitt, 1974) have attempted to draw these two categories together.

Progress to a full understanding of cheese flavour development has been slow for a number of reasons, among the most significant being: the number of cheese varieties known, the large number of different microorganisms and enzymes potentially involved, variations in the perception of acceptable flavour within a cheese variety, and the complexity and variability of the substrate (milk).

Basic Cheese Flavour. The flavour of all cheeses is based on a background composed of fat, protein, salt, and lactate along with the sensation of the texture of the cheese in the mouth. This background will include flavours entering with these components (e.g. feed flavours) and the effect of differences in texture (particularly differences in the moisture and fat contents). Overlaid on the basic flavour are the characteristic components not present in the fresh curd, which develop during maturation. The development of these components is catalysed by enzymes in the milk or from the added bacteria, and the rate of reaction is dependent on the environment created and maintained in the cheese block (salt and moisture contents, pH, storage temperature, humidity and packaging).

Three components provide the sources of developed flavour compounds- fat, carbohydrates/lactate and protein. Not all flavours which can develop are acceptable, and components which when dominant may be considered off-flavours, may be a required component at a lower intensity (the "Component Balance Theory" of Mulder, 1952, and Kosikowski and Mocquot, 1958). Indeed the perception of how intense a flavour component may be before it becomes an off-flavour may differ between different consumers. This is particularly evident in the case of aged Cheddar cheese (epicure) which contains intense sulphurous flavours not acceptable in mild cheese of the same variety.

The Development of Flavour in Cheddar Cheese.

Fat Metabolism. Metabolism of fat provides characteristic flavours in blue cheese (mould metabolism) and some Italian cheeses (added enzymes). However, fat is not believed to contribute directly to the development of flavour of most cheeses. Milk fat appears to contribute precursors for methyl ketone production but free fatty acids longer than C4 do not appear to contribute to cheese flavour (Lawrence, 1967). Ohren and Tuckey (1969) noted that cheese made from skim milk did not develop flavour. This has been confirmed by Deane (1972) and Deane and Dolan (1973). Foda et al. (1974), found some flavour to be reintroduced when synthetic fat or mineral oil were included in milkfat-free cheese. They suggested that one role of the fat may be to dissolve and hold non-aqueous flavour compounds.

The extent to which lypolysis is required in the development of Cheddar cheese flavour is still unknown. From research into enzymatic methods of accelerating flavour development, the presence of a low level of a lipase with specificity for medium length fatty acids appears beneficial (Arbige et al., 1986). However, if too much lipolysis occurs, the cheese are downgraded as rancid or with butyric acid flavour (at least some lipases preferentially produce free C4 acids from the mixed fatty acids present in butterfat).

Carbohydrate Metabolism. The primary effect of the starter bacteria added to milk for cheese manufacture is to convert the milk sugar (lactose) into lactic acid, thus protecting the

milk from putrefactive fermentations. Since the body of Cheddar cheese is expected to be uniformly close, without gas formation, only homolactic starter cultures are added. Therefore the lactose in milk is converted almost entirely to lactic acid. Only in cheese in which the starter failed to make acid during manufacture or in cheese with very high salt levels will significant levels of lactose remain after a few days (Turner and Thomas, 1980; Thomas and Pearce, 1981).

Further metabolism of lactate does not occur in Cheddar cheese since there is no added secondary flora. However the NSLAB bacteria which grow in all maturing cheese convert the L-lactic acid to a racemic mixture of lactic acids.

Thus, in Cheddar cheese, carbohydrate conversion to products other than lactate will not be a contributor to the development of flavourful compounds. However, an open question relates to the possible utilization of carbohydrate components of dead starter cells. The possibility that NSLAB may produce flavourful compounds from these substrates has yet to be addressed.

Heterolactic starters (the "aroma producers") are not used for Cheddar cheese manufacture since these organisms also produce gas and lead to an open cheese texture (Sherwood, 1939a; Hoglund, Fryer and Gilles, 1972a; L.E. Pearce, 1977). As a consequence, the characteristic Cheddar cheese flavour will not contain compounds associated with the metabolism of these starters (and required as a component of the flavour of

many cheeses). The role of aroma starters in the development of flavour in other cheese varieties will be reviewed in the introduction to Part III of this thesis.

Proteolysis in Cheddar Cheese Ripening. It is universally agreed that the breakdown of the milk proteins occurs in parallel with the increase in cheese flavour. The four main sources of enzymes which could be implicated in proteolysis in cheese are:

i) Pasteurization resistant enzymes from the raw milk or the raw milk flora;

ii) Enzymes from the starter;

iii) Enzymes from the rennet;

and iv) Enzymes from the secondary flora of microorganisms growing in or on the cheese.

The respective roles of these enzymes is uncertain, and will depend to some extent on the type of cheese in question.

In Cheddar cheese it is believed that the raw milk flora is of little effect unless the contamination is very high, and that the pure calf-rennet solutions currently in use in N.Z. are of such high purity that they contain only chymosin (>93%) and pepsin (<7%). Therefore, the pool of free enzymes is relatively small.

The starter cultures used in N.Z. consist of pure strains of *Streptococcus cremoris* organisms and not the mixed cultures (containing *Streptococcus lactis* plus other organisms) common elsewhere. Thus the pool of starter enzyme types in the cheese is relatively small.

The secondary flora of Cheddar cheese is composed almost entirely of lactobacilli and pediococci, commonly called the non-starter lactic acid bacteria (NSLAB). Therefore, unlike other cheese types where moulds, surface smear bacteria or internal bacteria (e.g. propionibacteria) compose the secondary flora, in Cheddar cheese, the pool of enzyme-types from the secondary flora is relatively small.

Therefore, research into the contribution of these various enzyme sources to the basic mature flavour of cheese should be more easily interpreted using N.Z. Cheddar cheesemaking conditions than by using other cheese types or starter systems.

<u>The Role of Bacteria in Cheese Ripening</u>. The two main contributors of enzymes which catalyse the development of cheese flavour are considered to be the starter and the NSLAB organisms in the cheese. Three groups of workers in particular have attempted to determine the role of starter bacteria in the development of cheese flavour using aseptic cheesemaking vats to control the bacterial flora. The groups at the U.K. National Institute for Research in Dairying, and the New Zealand Dairy Research Institute, worked with Cheddar cheese, while the group at Wageningen University worked with Gouda cheese. Both the N.Z. and Dutch researchers were particularly interested in the formation of bitterness in the cheese which was at the time a major defect.

The reports of all three groups agree that starter only is sufficient to produce cheese flavour. Both Perry and McGillivray (1964) and Lawrence (1966) claim lower flavour intensities (or slower flavour development) in cheese with starter only, but without presenting supporting evidence. Reiter (1973) commented "It has now been conclusively shown ... that starter itself produces cheese flavour." Lowrie et al. (1974) and Visser (1977) both noted that starters which did not produce the bitter defect were more capable of producing flavour than starters producing bitterness. The presence of bacteriophage for one bitterness producing starter (ML8) reduced bitterness formation and improved flavour production (Lowrie et al., 1974).

While starter alone may be adequate to produce cheese flavour, the NSLAB normally present in cheese may add overtones to the mature flavour (Lawrence, 1966). Reiter et al (1967) noted that the flavour of Cheddar cheese made in aseptic vats with no bacteria other than the starter, compared closely with that of cheese made in open vats. There was, however, a tendency for the cheese in the open vats to be described as fuller in flavour. When they added their "Reference Floras" during the aseptic manufacture of cheese, they noted that, at 6 months, cheeses without added lactobacilli had the most acceptable flavour while cheeses with lactobacilli present usually had off-flavours. They comment (without presenting evidence) that cheeses which had off-flavours at 6 months were "extremely strong and good

flavoured" at 11 months. Law Uet alu. (1976) could find no statistically significant evidence that cheeses containing lactobacilli were of different flavour to cheeses without these organisms. They do however note a trend to higher flavour intensities in the cheese containing lactobacilli. In contrast, Kleter (1976,1977) added three different lactobacillus cultures during aseptic manufacture of Gouda cheese and observed no increase in flavour intensity, but an increased incidence of "taste and flavour defects".

Aims of the Research Project. Starter alone may be sufficient to produce cheese flavour and different starters may differ in their ability to produce flavour. However, since the formation of flavour is still thought to be slower in the absence of NSLAB, it is important to provide stronger evidence of the potential beneficial effects of these organisms in cheeses made under strictly controlled conditions. Furthurmore, since no previous research on cheeses made in aseptic vats has involved maturing the cheeses in bags (and virtually all semi-hard cheeses are now matured in bags), it was important to determine whether this form of maturation influenced the results obtained.

Some measure of the metabolic activity of the NSLAB present adventitiously in Cheddar cheese can be gained from the observation that the L-lactic acid produced by the starter is rapidly converted by the NSLAB to the racemic DL mixture, a modification of approximately 0.7% of the cheese weight

(Turner and Thomas, 1980; Thomas and Crow, 1983). Recent observations (Fryer, pers. comm.) have indicated that in slow-cooled Cheddar cheeses the presence of heterolactic lactobacilli correlated strongly with the presence of fermented flavours at an early age. Lactobacilli not normally considered to be heterolactic can also produce potentially aromatic products under conditions likely to be encountered in cheese (Thomas, 1986). At the very least, NSLAB are active in cheese, and can produce off-flavours if present in high enough concentrations.

NSLAB occur in all cheese made in normal vats. The only methods available to prevent their growth are either the use of selective antibiotics, or their total exclusion during manufacture. Since the use of a selective antibiotic which did not inhibit starter bacteria (e.g. a starter-produced bacteriocin) was not possible, the construction and use of totally enclosed and sterilized cheesemaking vats was considered the most appropriate method of obtaining NSLAB-free cheese.

PART I. METHODOLOGY

In order to obtain cheese without NSLAB or with no NSLAB other than known strains deliberately added (and to determine their effect on the cheese flavour) it was necessary to design, build and commission a suitable cheesemaking plant.

The major section of Part I (Section I.A) describes:

the design and commissioning of the vats (I.A.1);

the initial manufacture of cheese in the vats using pasteurized milk that had not been aseptically drawn (I.A.2);

the determination of the thermal death characteristics of NSLAB and proof of the need for aseptically drawn milk (I.A.3);

and the proof that aseptically drawn milk did not contain NSLAB (I.A.4).

The second section (Section I.B) describes the development of a trained panel to determine the flavour and texture of Cheddar cheese by attribute scaling methods.

The detailed design drawings (Figures 1-6) were drawn by Mr. I. Horley of the N.Z. Dairy Research Institute, and the construction undertaken by the NZDRI workshops. As project leader, the candidate was involved in a supervisory role in all stages of the design, construction and installation of the vats.

I.A.1 The Design and Operation of the Aseptic Vats.

I.A.1.1 Introduction. During the last three decades, there have been a number of reports describing research projects using cheese made under "aseptic" or "controlled microbiological" conditions. Most designs were for small scale equipment (2-20 L) to be used in the laboratory (Ohmiya and Sato, 1970; Le Bars et al, 1975; O'Keefe et al, 1975, 1976a,b; Singh et al, 1976; and Paulsen et al, 1980). These have been useful in studies involving specific biochemical questions relating to the role of starter bacteria in proteolysis and lipolysis. None produced sufficient cheese for adequate flavour analysis during maturing.

Two groups have designed larger equipment producing sufficient cheese to allow for extensive flavour analysis. Kleter (1975) developed a 40 L system, while workers at the National Institute for Research in Dairying developed 50 and 160 L systems (Mabbitt et al, 1959; Chapman et al, 1966; Law et al, 1976a,b, 1979). The Dutch and the later and bigger N.I.R.D. system both involved autoclaving to sterilize the vats.

A number of early reports indicated that the manufacture of "NSLAB-free" cheese from pasteurized milk was possible in vats which were not autoclaved (Mabbitt et al, 1959; Perry and McGillivray 1964; Lowrie et al, 1974). Furthermore, autoclaving of vats large enough to produce more than 4 kg of cheese requires both a large autoclave (not available at

N.Z.D.R.I.) and the use of materials which tolerate the high sterilizing temperatures (particularly in gaskets, seals and gloves). Therefore, it was decided to design a pair of vats of 75 L capacity which could be sterilized in place by chemical soaking followed by steam flushing.

I.A.1.2 Design Criteria Selected. The criteria selected as the basis for designing the aseptic vats are set out in Table i. The aim was to manufacture cheese under conditions as close as possible to current N.Z. Cheddar cheese manufacturing practices in order to ensure that the results obtained would be as relevant as possible to the current situation.

I.A.1.3 Description of the Plant. The design of the vats is shown in Figures 1-6. Two vats were built as mirror images, mounted with a common entry/exit pipe entering at the bottom of the vats to minimize pipe length (Fig 1). All the parts of the vats, pipes and fittings in contact with the product were manufactured from grade 316 stainless steel. The pasteurizer was mounted behind one vat and under the shelf. The main controls were mounted between the vats at operator level. These included switches for all the motors, the programmable controller which controlled the temperature of both vats and the pasteurizer, and the switches for the pneumatic valves on the vat entry/exit pipe.

Table i. Design criteria for aseptic cheesemaking vats.

i) A totally enclosed system capable of being operated in a dairy plant environment with the required services (hot and cold water, steam, air, electric power).
ii) Two vats, preferably side by side.
iii) Suitable for the manufacture of Cheddar cheese with cheddaring to be done in the vat. However, flexibility to be maintained so that brine salted varieties could be made by addition of appropriate modules.
iv) Minimal complexity to ensure the least likelihood of contamination or operator error.
v) Operator to be able to reach all internal surfaces (through gloves).
vi) Maximal working volume (given Criterion v) to maximize the weight of cheese produced.
vii) Sufficient mechanization, especially of curd agitation and temperature control, to ensure the closest similarity between operations in each vat and to reduce day to day variations.
viii) Milk to be heated only to pasteurization temperatures so as to maintain the closest similarity to commercial practice.
ix) Cheese dimensions, handling and composition to compare closely to commercial practice. Cheeses to be matured in barrier bags.

The main body of the vats (Fig 2) was circular for ease of agitation. An operating volume of 75 L was chosen giving the vats a 500 mm internal diameter with a 400 mm operating height. With gloves mounted in a sloping front piece, the operator could reach the bottom far edge of the vats with very little effort. The provision of an enlarged shelf above the working height of liquid enabled small ancillary equipment to be stored in an area which the operator could easily reach. The vats were double jacketed with a spray ring (Fig 2) in the jacket to enable heated water to be circulated for heating the vats.

The operations could be viewed through a Lexan (General Electric Co., Pittsfield, Ma., USA) porthole installed in the front of the vats above the glove ports (Fig 2). Together with the lexan cover at the back of the shelf, these ports admitted sufficient light to be able to see the operations. The lexan cover at the back of the shelf was hinged to enable the door to be opened to admit the ancillary equipment or to remove the filled cheese hoops at the end of manufacture.

Details of the seals on the glove ports, viewing port and rear door are shown in Fig 3. The gloves (750 mm, Schack Industrial Rubber Gloves, Supreme Rubber Co, Auckland, N.Z.) were stretched over the flange (Detail B) and clamped by a stainless steel ring attached by three clips screwed into place. The viewing ports (Detail A) were similarly clamped with a stainless steel ring against the flange, but with a continuous silicone O-ring between the lexan and the flange. The rear door consisted of a rectangular piece of lexan screwed into a stainless steel frame. The frame was hinged to the flange of the vat (Detail C). The whole door was clamped closed during cheese manufacture by four hand tightened screw clamps which sealed the lexan to a continuous silicone O-ring in the flange (Detail D).

To agitate the vat contents, a drive unit was mounted on top of each vat (Fig 2). Each unit consisted of a 0.37 kW 1390 rpm TEFC electric motor (ASEA Type MBN 71 B4; Andrew Stewart Ltd., Palmerston North, N.Z.) attached by a belt drive to a 15:1 reduction unit (Penfold SP45, J. and A.P. Scott Penfold

Figure 1. The front (A) and side (B) elevations and the plan view (C) of the aseptic vats showing the location and elevation of the vats, control panel and electronics box, the pasteurizer and the piping and valves. The framing detail (D) shows the design of the mild steel frame on which the vats and equipment were mounted.

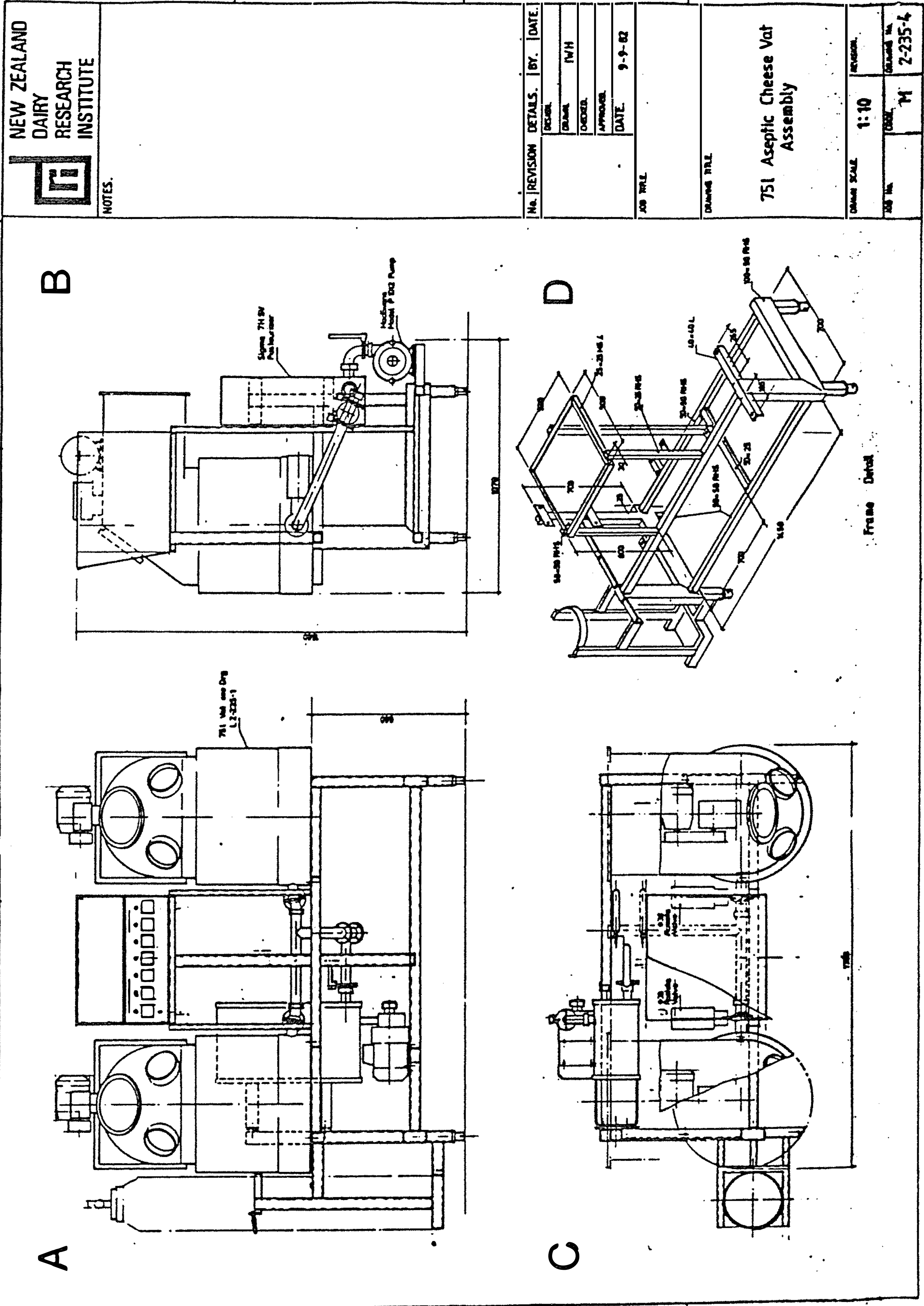
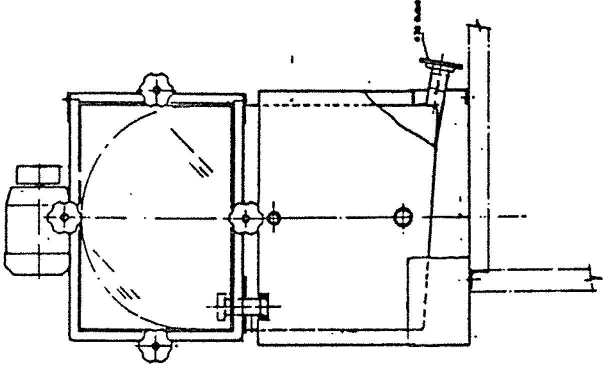
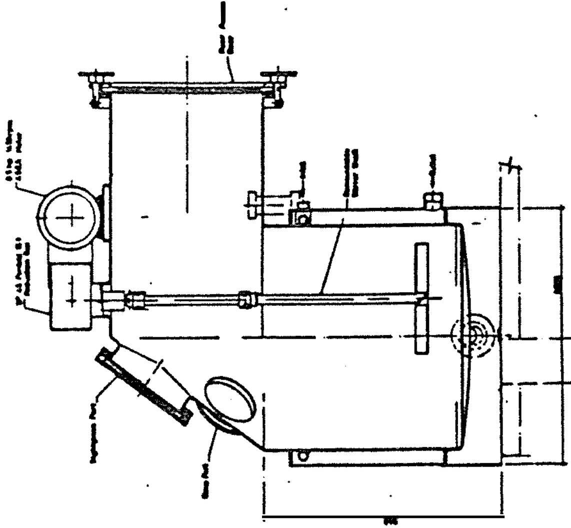


Figure 1. Front and side elevations of the aseptic vat unit, plan view and frame detail.

Figure 2. The side (A), rear (B) and plan views of the left aseptic vat showing the location of the porthole and glove ports, the agitator, its drive motor and reduction gearbox, the jacket and spray ring, the air inlet and outlet fittings, the sample bottle port, the starter inlet and the rear door.





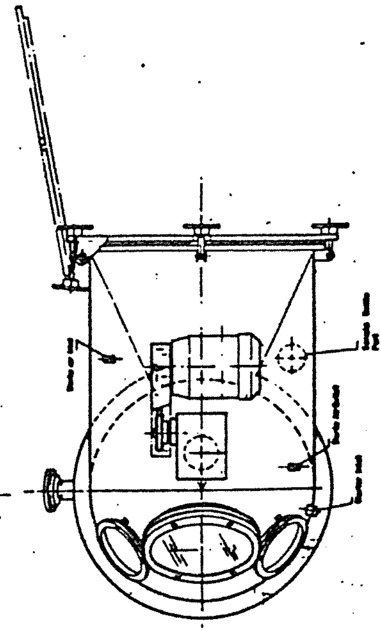


Figure 2. Side rear and plan views of details of each aseptic vat body.

Figure 3. Detailed drawings of the mounting details showing: the viewing port mountings (A); the glove attachment (B); rear door details (C) including expanded details of the hinge (C1), hinge location (C2), and swing bolts (C3); location of the starter port (D); detail of the sampling port (E); and detail of the overflow outlet in the water circulation jacket (F).

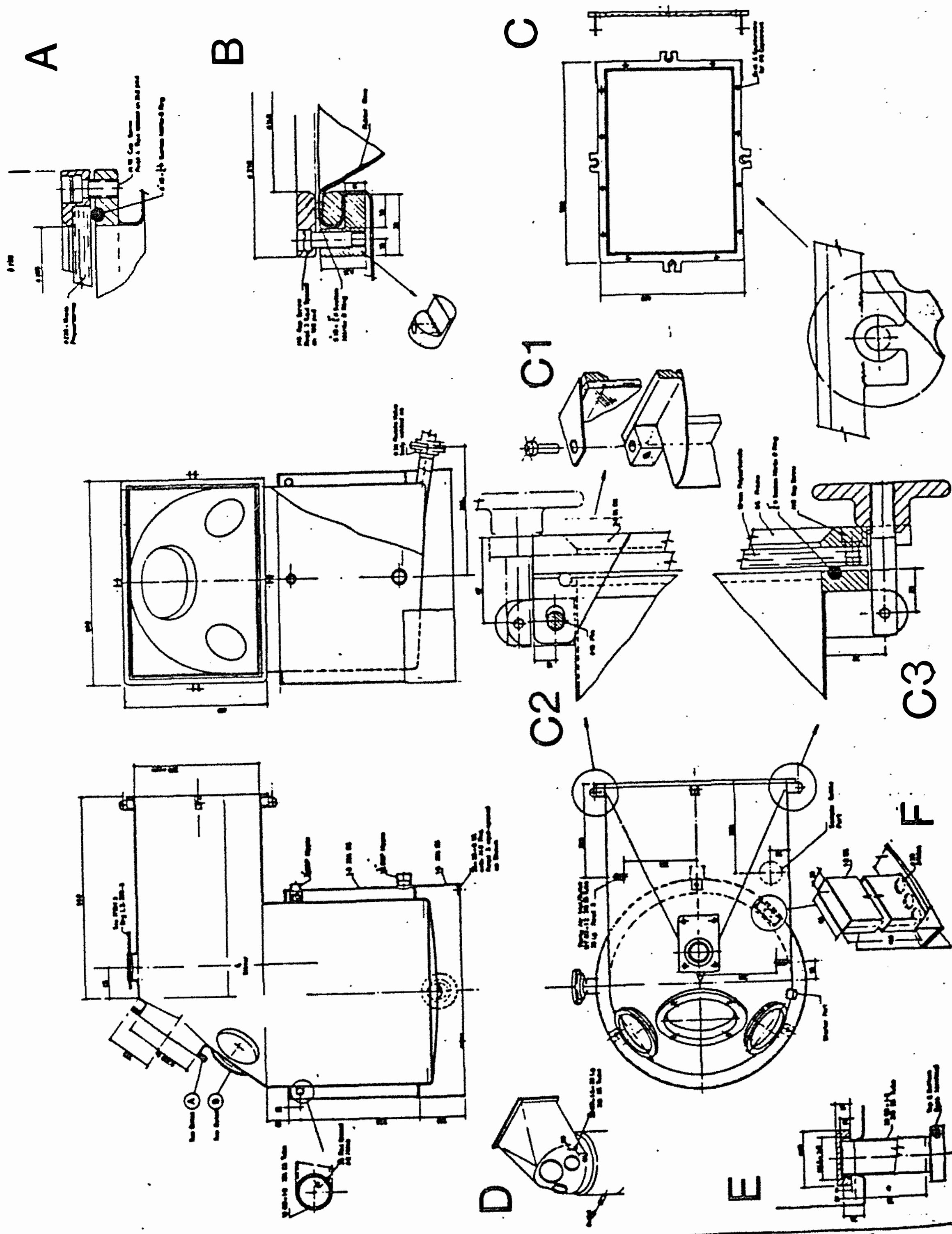


Figure 3. Details of all mountings on the aseptic vat bodies.

Figure 4. Details of the design of the agitator (A1), its mounting (A2), seals (A3) and the internal housing (A4); the male (B1) and female (B2) ends of the bayonet fittings and the blades (C1 and C2).

Figure 4. Agitator design and mounting detail.

Figure 5. The piping diagram for the aseptic vats showing elevation (A) and plan (B) views of the circulation piping for vat heating and pasteurizer heating and cooling, and the balance tank (C) for mixing hot and cold water in the vat heating circuits. Drawing D is a view of all the water circuits showing the location of all pumps, valves etc. including: 1. Pasteurizer primary heating control valve; 2. vat heating circulation pumps; 3. balance tanks; 4. pasteurizer heating circulation pump; 5. milk pump; 6. milk feed flow control valve; 7. manual valves; 8. pneumatic vat valves; 9. non-return valves; 10. Pasteurizer heating circuit flow control valve; 11. Pasteurizer; 12. Vat; 13. strainer; and 14. Hot water inlet solenoid valve.

Figure 5. Piping diagram for the aseptic vat unit.

Figure 6. Detail of the ancilliary cheese making equipment for the aseptic vats. The drawings show: the cutter screen in plan view (A1) and elevation (A2), the hinge (A3) and handle (A4); the curd mill (B); the outlet screen (C); the hoop (D), consisting of a lid (D1), the main body (D2) and the pressing frame (D3) into which the hoops were placed before pressing; and the sampling template intended to ensure accurate sampling of the cheese.

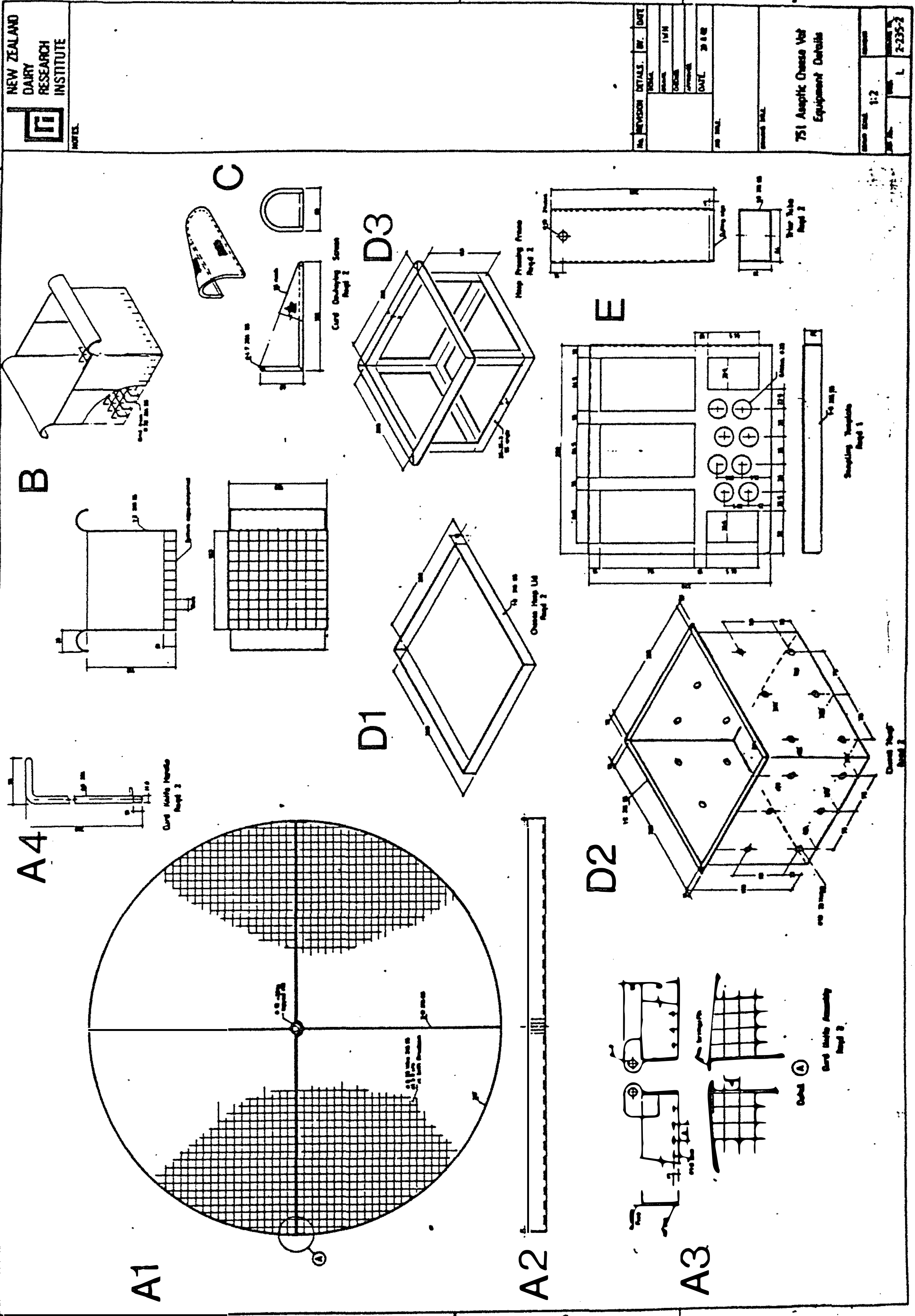


Figure 6. Detail of the ancilliary cheese making equipment for the aseptic vats.

Ltd., Christchurch, N.Z.). The drive shaft entered the vats through a water-lubricated Crane type II seal with a rotating carbon and stationary stainless steel face (John Crane Mauri Ltd.,Auckland, N.Z.; Fig 4) and was terminated with a bayonet fitting. The agitator shaft was made in two parts with a bayonet fitting just above the liquid level (Fig 4). The four blades of the agitator were welded at an angle of 45 to the bottom of the lower half of the shaft. The agitation speed in the vat was further reduced to 75 rpm by a change in the size of the pulleys during commissioning.

The vats were heated by circulating heated water through the spray ring. A platinum-bulb thermistor was attached in a pocket to the bottom of each vat and connected to the temperature controller. The piping of the vats is shown in Fig 5. The controller opened a solenoid valve (B8, Bestobell Ltd., N.Z.) allowing hot water (approx. 70 C) to enter the water circuit. The water was continuously pumped by a 0.1 kW circulating pump (Commodore, NDA Ltd., N.Z.) through the spray ring. The water then returned to a balance tank by gravity. Cold water (at 2 L/min) was continuously fed into the balance tank to reduce temperature overshoot, and surplus water ran to waste through an overflow. The tank provided the continuous head to the circulating pump.

The pasteurizer was a 600 L/h Sigma 7H unit (W.Schmidt GmbH, Bretten, West Germany). Incoming milk was pumped by a 0.56 kW centrifugal pump (P1012, MacEwens Machinery, New Zealand) throttled by a manual Rosista butterfly valve (Holstein und

Kappert GmbH, Unna-Konigsborn, West Germany) and warmed against the outgoing milk, before entering the heating section. A platinum bulb thermistor was inserted in a pocket in the external pipe linking the holding and regeneration sections and linked to the temperature controller. Heating was by superheated water (boiler primary supply) admitted to a closed circuit by a pneumatic Transflo Control valve (Taylor Instruments, Wellington, N.Z.) with an electric to pneumatic converter (Moore Products Ltd., Surrey, England). A 0.25 kW centrifugal pump (K-series with gunmetal impellar, Davis Pumps, N.Z.) maintained circulation in the loop. Excess water was removed by a pipe to the boiler return line just before the pump inlet. A tap in the circuit was manually set to maintain the required pressure differential between the supply and return water lines.

The computer to control the time and temperature in the vats and in the pasteurizer was built and programmed by staff of the DRI Applied Mathematics Section.

With the temperature probes and electrical wiring attached to the vats and also the physical size of the apparatus, autoclaving was not possible.

The vats were supplied with pressurized air through a pressure reducer (Martonair, Twickenham, England). The air was used to raise the Taylor valve in the pasteurizer hot water circuit and two pneumatically operated Rosista butterfly valves on the outlet pipes of the vats (Fig 1). The butterfly valve for each vat could be raised by a manual switch mounted

below the main control panel, or by a foot pedal (S31-FS, Kenny Pneumatics Ltd., Palmerston North, N.Z.) attached to the base of the frame allowing the operator to open the valve while working with his hands inside the vats.

The air was also used to maintain a positive pressure within the vats. A line to each vat was controlled by a small Martonair regulator mounted below the control panel. This line fed through presterilized air filters (Whatman Gamma 12, W and R Balston Ltd., Kent, England) into the vats through the fittings attached to the roof of the vats (Fig 2). A similar filter was attached to an outlet fitting to enable air to flow through the vats.

Various small ancillary items which were also required are shown in Fig 6. These included a cutting screen to cut the coagulum, a fine meshed screen to prevent the curd escaping down the outlet during whey removal, a mill and a hoop, along with sample containers and salt. The cutter was designed to be left in the bottom of the vat before renneting, drawn up through the curd, and then folded and shelved. However it was not possible to obtain sufficient strength on the rim to hold the shape without making the rim unreasonably thick. Instead, by stringing the wires in only one direction, the light rim was sufficient to hold the cutter in shape. The coagulum was cut by passing the cutter down through the curd, rotating it through 90 degrees and raising it again. This proved adequate to cut the coagulum into rectangular columns approximately 9 mm by 9 mm.

The curd screen consisted of a stainless steel mesh soldered to a stainless steel rim shaped to fit over the gullet in the bottom of the vat during whey removal.

The mill was designed as a stainless steel box with a sharpened grid (12 mm apart) welded into one of the open sides. It was found to be totally impractical as it was not possible to push the mill through the cheddared curd. Instead, the curd was milled by cutting with a stainless steel cooks knife with a heat resistant plastic handle.

The hoop was a stainless steel rectangular box with dimensions scaled in proportion to those of the standard N.Z. 20 kg cheese block. The design capacity was 7.5 kg of pressed cheese. A cloth bandage was sewn as a square bag to accurately fit the inside of the hoop. After filling the bandaged hoop with curd, the bandage flaps were folded over the top and a stainless steel lid placed on top.

The front and rear views of the vats in place in the processing plant are shown in the plates (Plate 1 and Plate 2).

I.A.1.4 Plant Sterilization. On the afternoon before cheesemaking, the vats (with the gloves attached and the rear doors secured) were filled with water containing 1.6 mL of Iodophor (Economics Laboratories, Hamilton, N.Z.) per litre. The pasteurizer pump was used to pump the entire headspace

Plate 1. Front view of the aseptic vat unit installed in the dairy plant.

Plate 2. Rear view of the aseptic vat unit installed in the dairy plant.

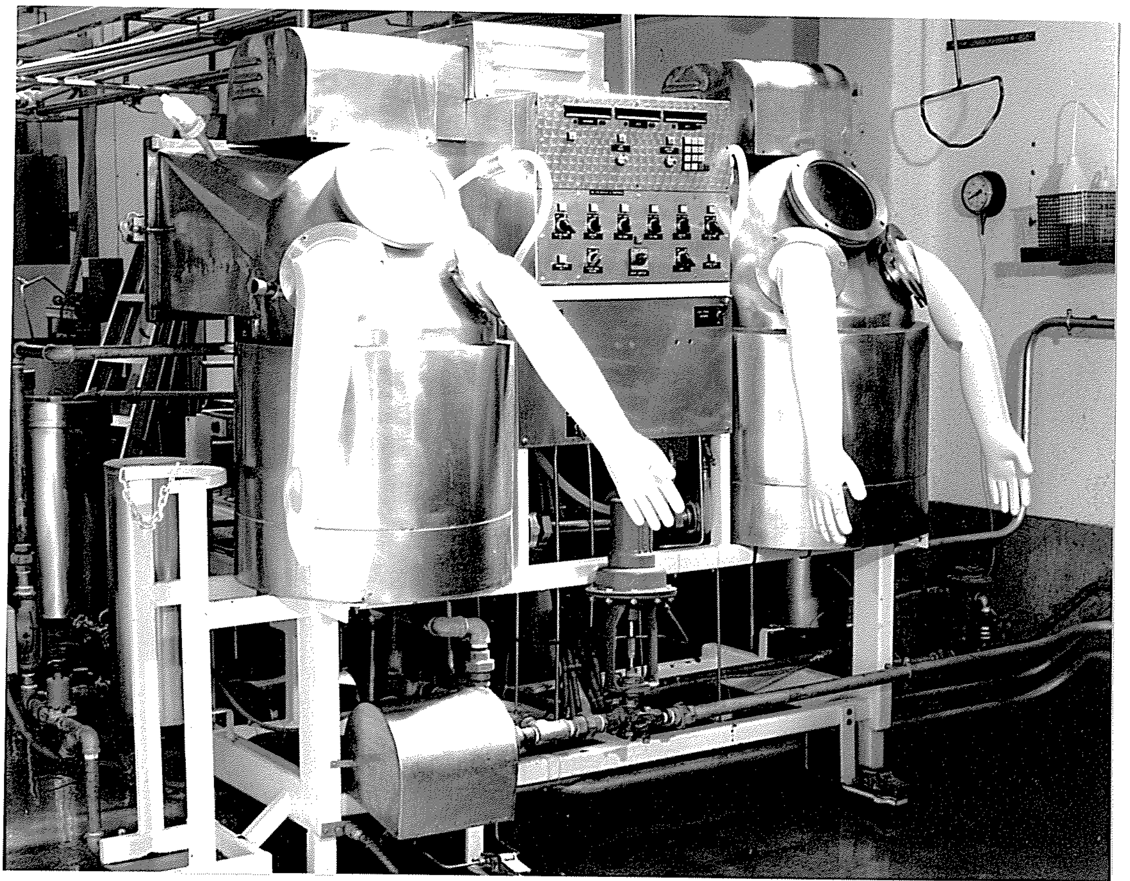


Plate 1

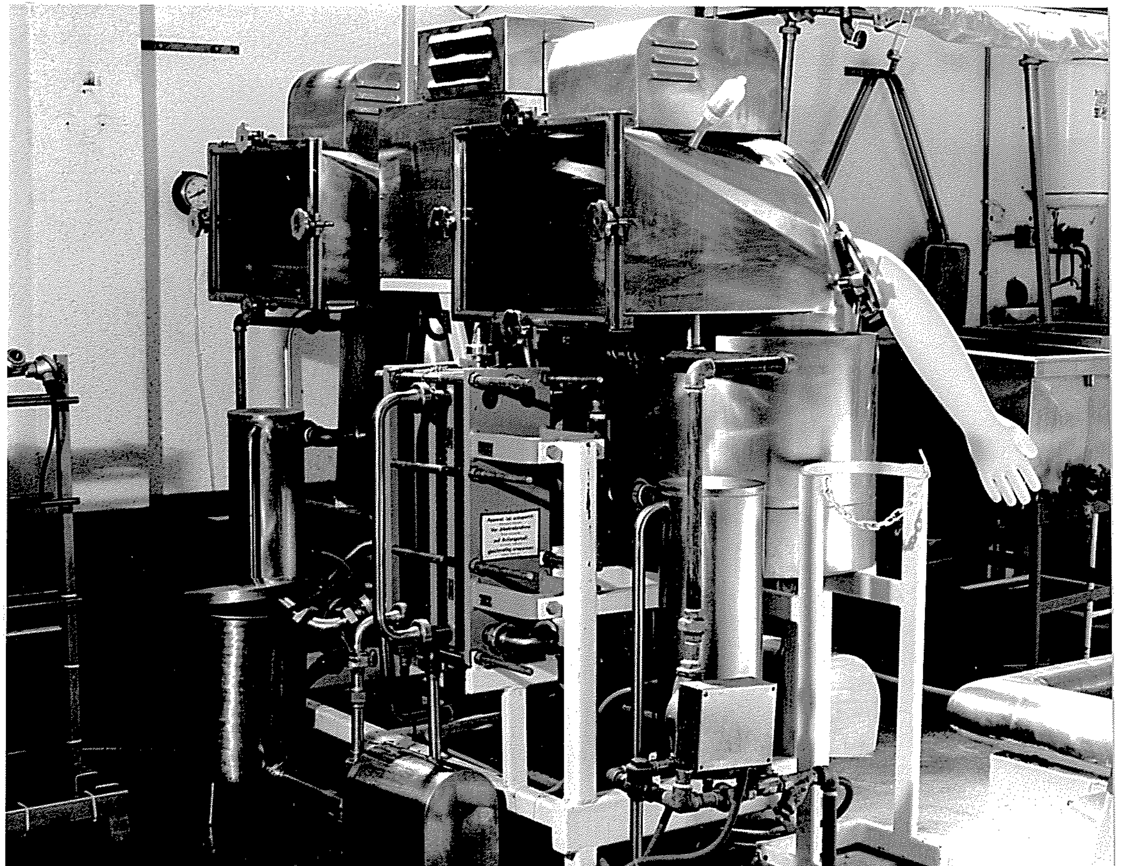


Plate 2

full of Iodophor. Thus, the pasteurizer, pipes and balance tank were all filled with Iodophor which was left in the plant overnight.

In the morning, the sanitizing solution was drained and the vats heated with steam at atmospheric pressure. The steaming was maintained for at least 1 hour from the time that the vat thermometers registered 95 C. During the steaming period, the valves on the inlet/outlet pipes were left slightly ajar so that steam vented through the outlet and sterilized the pipes. After about 30 min steaming, the presterilized air filters were attached to the plant.

All the small ancillary equipment was autoclaved (121 C/15 min) prior to use, wrapped in Kraft paper and secured with autoclave tape. The agitator shaft, screen, knives, hoop lid, sample containers, and salt (weighed into 250 mL polycarbonate Erlenmeyer flasks) were autoclaved inside each vat's hoop. The cutter, agitator paddles and hoop bandage were autoclaved separately wrapped. After a total of at least 45 min of steaming, and while steam was being supplied, the presterilized small equipment was passed into the plant through the rear doors. As each package was passed in, the paper was removed. Care was taken not to touch the sterile equipment either with hands or against surrounding nonsterile surfaces.

Following entry of the small equipment and a further 15 min of active steaming, the air supply was connected through the filters, the steam disconnected and the vats allowed to cool

to ambient temperatures with a positive supply of sterile filtered air. The air supply was regulated to maintain the gloves in a slightly inflated state so as to prevent non-filtered air being sucked into the plant.

I.A.1.5 Sterility of the Plant. The sterility of the plant was checked in two ways.

I.A.1.5.1 Swabbing. After a number of manufacturing runs, the plant was sterilized as described in Section I.A.1.4 and then carefully stripped down and swabbed. Attention was paid to areas like butterfly valves, O-rings, glove attachment sites and the agitator drive bearing, where non-sterlie buildup may occur (Fig 7). The swabs were streaked onto pre-poured plates of Standard Methods agar (SMA) and Lactobacillus selection agar (LBS) agar and incubated. No NSLAB contamination was found, but as a contaminant which grew on SMA was found in the air intake on one of the vats, the filters were changed to Whatman Gamma-12 filters which are fully autoclavable.

I.A.1.5.2 Incubation of UHT Skim milk. The plant after sterilization, was filled to operating volume with UHT-treated skim-milk and held at 30 C.

I.A.1.5.2.1 Preparation of the UHT Milk. The UHT skim milk was prepared by first heating through the UHT plant at 115 C for 3 s, cooling and holding overnight at 10 C to allow for the germination of spores. The milk was then heated in the sterilized plant at 145 C for 5 s, and pumped from the outlet

through a sterile silicone tube terminated with a shortened syringe needle, through the Suba-seal into the sterilized plant.

I.A.1.5.2.2 Incubation of the UHT Milk. The milk was temperature controlled at 30 C during the day and left quiescent at night. During the entire incubation period, the filtered air supply to the vats was maintained. The microbiological counts and pH values of the milk are shown in Table ii. After a period of 44 h the milk pH started to fall as a result of the growth of bacillus organisms. No NSLAB organisms were detected in the vat milk throughout the entire incubation run (up to 90 h).

Samples of the UHT milk taken from the delivery stream within the vat but without contact with the vat surfaces did not sour, even after incubation at 30 C for a number of weeks, indicating that the contamination did not occur in the UHT milk itself.

Table ii. The microbiological counts and changes in pH of UHT treated milk incubated in the sterilized aseptic vats.

Sampling time (h)	Bacterial count (cfu/mL) Aerobic	Anaerobic	pH
0	<2	<2	6.62
6	<2	<2	6.60
22	2	<2	6.60
28	102	<2	6.55
44	$1*10^4$	100	6.28
50	$5*10^6$	200	5.95
66	$1*10^8$	$1*10^7$	5.04
90	$1*10^8$	$1*10^8$	4.90

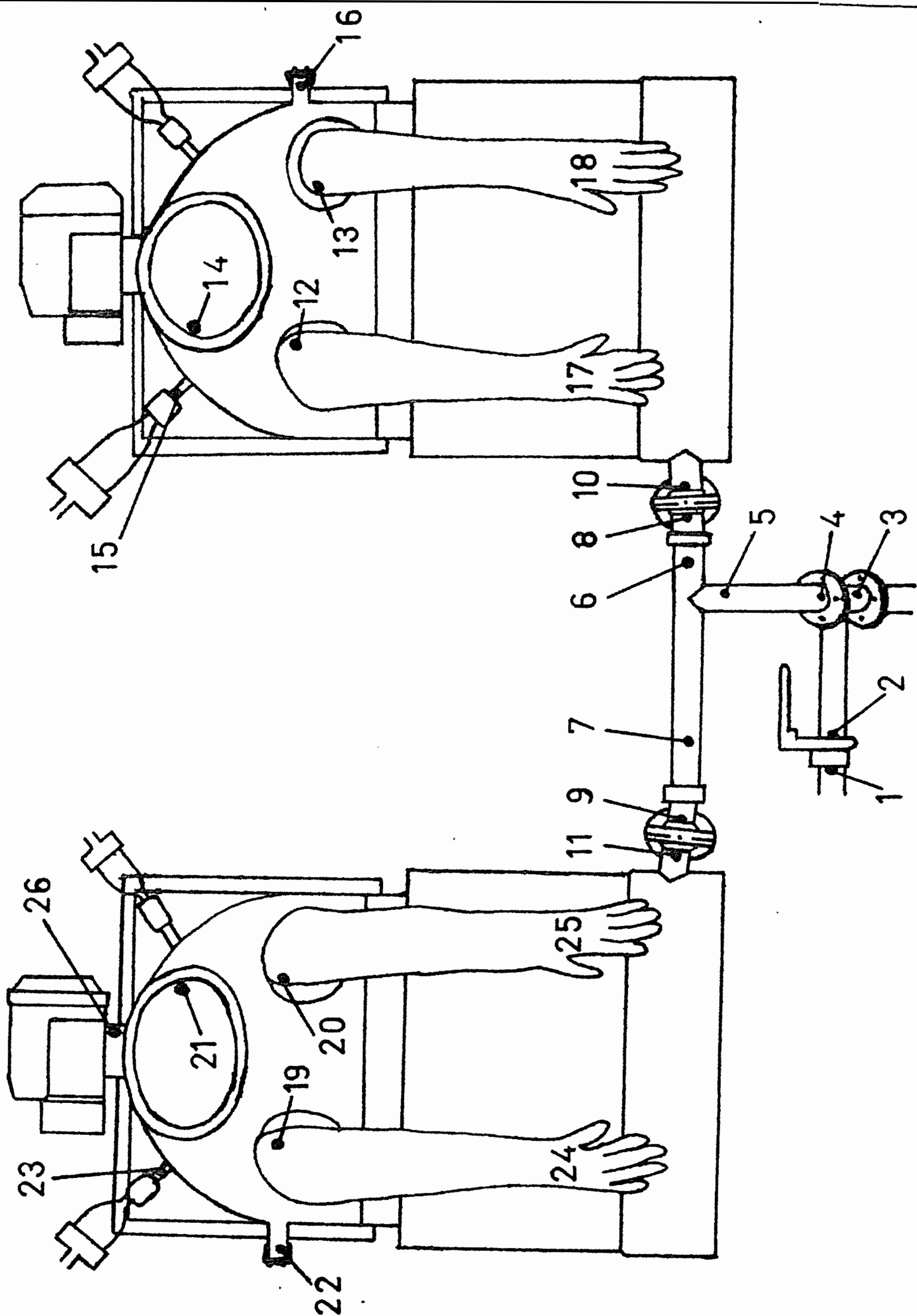


Figure 7. Location of sites for swabbing of the sterilized aseptic vats. After sterilization, the aseptic vats were carefully stripped and swab samples were taken at the sites indicated. Samples were plated on both standard methods agar to determine the total count of bacteria, and on lactobacillus selection agar to detect the presence of NSLAB.

I.A.1.6 Pasteurization and Milk Sampling. The pasteurizer was operated at 73.5 C with a holding time of 17 s. It was run up to operating conditions by recirculation of the Iodophor-treated soak water after steaming was complete. The whole of the pasteurizer was allowed to come to temperatures around 80 C before the cooling water was turned on and the inlet throttling valve closed down to the operating flow rate. The circulating water was then cooled to around 30 C. After the plant had attained stable operating conditions, milk pasteurization was commenced by turning the circulating water to waste. Milk was poured into the balance tank once the water was removed.

Samples for microbiological and chemical assessment were taken from the cans of milk with a sterile dipper, after the milk had been well mixed using a sterile plunge stirrer. Proportional volumes were taken from each can and mixed in the sample container.

The milk was poured from each can into the pasteurizer balance tank. The first 45 s of milk through the pasteurizer was run to waste and the pasteurizer holding temperature was monitored. In the event that the temperature fell below 72 C (due to the circulation water and the milk being of different temperatures) the milk was run to waste until 45 s after the temperature returned to above 72 C.

Both vats were filled together, the filling time being measured to ensure that the correct holding times were obtained. At the time of commencement of filling, at

approximately half way, and immediatly after the vats were full, samples of the pasteurized milk were obtained from the outlet pipe for microbiological assessment. The milk in the vats was also sampled for microbiological assessment before the addition of starter.

I.A.1.7 Cheese Manufacture. The manufacturing protocol for Cheddar cheese manufacture is outlined in Table iii which gives the fixed time and temperature points used by the computer programme to control the temperature in the cheese vats. As soon as enough milk had been filled into vat 1, the agitator was attached, the temperature programme to that vat was switched on and the manual override valves controlling the supply of hot and cold water to the temperature circuit were opened. The control programme contained an initial 25 min holding period to allow for final equilibration at the setting temperature. The temperature programme and agitator for vat 2 were switched on 15 min after vat 1 so that the operations in the two vats were staggered 15 min apart.

At 25 min, the starter was added by inserting the needle attached to the bung on the starter flask through the Suba seal of the vat. Prior to insertion of the needle, the Suba seal was sterilized by wiping with alcohol. The starter was pumped into the vat by pressurizing the flask with filtered air.

Table iii. Cheesemaking protocol for Cheddar cheesemaking in the aseptic vats.

Time(h.min)	Temperature(C)	Comments
0.00	32.0	Initiate equilibrium
0.25	32.0	Add starter
0.30	32.0	Add rennet, cease stirring close manual hot and cold valves
1.10	32.0	Cut coagulum, take whey sample, start agitation
1.15	32.0	Commence heating
1.50	38.0	Cease heating
3.20	37.0	Commence running whey take whey sample
3.25	37.0	Whey removal complete, dry stir, take whey sample at outlet, commence Cheddaring, close manual water valves
5.00	approx 35	Commence milling
5.10	approx 35	Take pH sample, add salt
5.50	approx 33	Commence hooping

At 30 min, rennet (at the rate of 16 mL/100 L) was injected through the Suba seal in the side of the vat. Normal strength (59 Ru/mL) NZ calf rennet (New Zealand Cooperative Rennet Co, Eltham, N.Z.) was filled into a plastic syringe and injected through a presterilized Swinex 25 filter unit (Millipore Corp., Mass., USA) equipped with a 0.45 u Millipore membrane filter (Type HA). Again, the outside of the Suba seal was swabbed with alcohol prior to insertion of the needle.

After 2-3 min stirring to ensure adequate mixing, the agitators were stopped, disassembled and shelved. The hot and cold water to the heating circuits was turned off since the

temperature probes were attached to the vats not the water balance tanks, and therefore temperature control was lost when agitation ceased.

Forty minutes were allowed for coagulation and curd firming. The cutter was then opened and passed down through the coagulum, turned through 90° at the bottom and raised, folded and reshelved. The agitator was reassembled and after allowing 2-3 min for initial whey expulsion (and removal of a whey sample) agitation and temperature control were recommenced. A combination of manual and mechanical agitation was used to move the curd which was cut into cubes of approx. 10 mm by the agitator blades. After approximately 10 min, the whey volume was sufficient that the agitator could maintain the curd particles in suspension.

Automatic temperature control was maintained through the heating (cooking) and holding periods until the elapsed time reached 3 h 20 min (i.e. 2 h 50 min from rennet addition).

Agitation and temperature control was stopped approx. 5 min before running the whey. This allowed the curd to settle and made the separation of curd and whey easier. After taking a whey sample, the curd was gently pushed back from the outlet and the draining screen inserted over the gullet. The airflow was increased to maintain a positive air pressure and the pneumatic butterfly valve was opened using the foot pedal. Flow through the outlet was regulated by intermittantly closing the valve. As required, the curd was cleared from the screen by hand.

Once the whey was removed, the curd was dry stirred three times, working around the circumference of the vats. The curd was piled around three-quarters of the circumference of the vat (away from the outlet) and a sample of whey from the outlet pipe was taken for acidity determination. After 5-10 min, the curd was trimmed using the milling knife and after another 20-25 min, the curd was cut into 8 approximatly equal blocks for cheddaring. The Cheddar blocks were turned approximatly every 15 min. During cheddaring the outlet valves were normally open allowing the whey to run to waste, and the air flow was maintained to sweep filtered air through the outlet pipes.

Care was taken to ensure that whey was not forced from one vat into the other. The outlet valves were closed when necessary (e.g. on vat 1 during removal of whey from vat 2 at running) and air flows were adjusted to equalize pressures in both vats before reopening the valves.

At 5 hours elapsed time (4 h 30 min after rennet addition) the Cheddar blocks were cut into curd fingers (approx. 10*10*150 mm), using the milling knife. This, being entirely manual, took 8-10 min per vat. After thorough mixing, 3 or 4 curd fingers were forced into a sample bottle for pH measurement, and the remainder mixed with salt poured out of the Erlenmeyer flask stored inside the hoop. The curd was mixed again after 10-15 min and hooped after 20-25 min. The closed hoop was placed at the back of the shelf.

The curd was pressed outside the vats. The rear door was opened and the hoop carefully placed into a large cheese bag (P100, Wrightcels Ltd., Feilding, N.Z.) taking care that the hoop only touched the inside of the bag. The hoop, shrouded in its bag, was then placed in a vertical cheese press and pressed overnight at a pressure of 400 kPa using a 150 mm diameter ram. The cheese bandage was not tightened around the cheese (dressed) after pressing was commenced.

I.A.1.8 Vat Cleaning. Following removal of the filled hoops, the plant was rinsed with cold water to remove any remaining whey and curd particles, and the gloves were removed. The cutters and screens were placed on the bottoms of the vats and the agitators assembled. Caustic cleaner (1.5%) and caustic surfactant solution (Klenzade, Economics Labs., Hamilton, N.Z.) were added to the vats and pasteurizer balance tank which were then filled with water at around 70 C to levels just above normal operating levels. The vat agitators and the pasteurizer pump were activated and the cleaner left circulating for at least 1 h. The caustic solution was run to waste and the vats and pasteurizer thoroughly rinsed with cold tap water. Areas of the vats not reached by the caustic solution were scrubbed with soapy water and then both the inside and outside of the vats was hosed with a steam and water mix at around 100 C from a steam hose. The outlet valves were left raised until the vats were dry. The gloves were washed separately in warm soapy water, rinsed and dried.

After the cheeses were removed from the hoops, the hoops were scrubbed with warm soapy water, steamed and allowed to dry. The bandages were washed separately in warm soapy water, rinsed and dried.

I.A.1.9 Dehooping, Bagging and Maturing. After overnight pressing, the cheeses were dehooped and carefully placed inside cheese bags (P60, Wrightcels Ltd., Feilding, N.Z.) so that the cheeses touched only the inside of the cheese bag. The cheeses were then moved to the laboratory and sampled using aseptic techniques. The bags were heat sealed under vacuum, placed in insulated cheese boxes and then into either the 10 or 15 C maturing rooms.

In experiments when half cheeses were matured, the dehooped cheeses were partially removed from their bags to enable a sterilized cheese wire to be drawn through the center of the block. The top half was then carefully placed into a second bag, again touching the cheese only with the internal surfaces of the bag. Both halves were then sealed under vacuum and placed in the maturing rooms.

I.A.1.10 Composition of the Cheeses. The compositions of cheeses made in each project section are given in those results sections. Initial manufacture in the vats was aimed at obtaining the optimum agitation speed to maintain the curd in suspension and minimise fat losses. The optimum speed was found to be 75 rpm, which maintained the curd in suspension

after some whey had been expelled (about 15 min after cutting). A combination of manual agitation and increasing proportions of mechanical agitation allowed the curd to become suspended with a minimum of fat loss and fines production.

The composition of the milk, the whey expelled to the drying stage, and the day old cheese from a typical manufacturing run is given in Table iv. This table also includes data obtained from an identical trial using milk of the same batch in one of the Institutes open vats (the full method of manufacture in the open vat is presented in Section II.2.2.3).

Since the same batch of raw milk was used to produce the pasteurized milk in both vats, there was no significant difference in the composition of the vat milks. However the whey from the aseptic vats contained twice the level of fat (0.65 against 0.34%) as did the whey from the open vat. The total solids difference of 0.32% reflected this difference in fat content. The mineral content of the wheys was little affected by the difference in fat content.

In the cheeses after pressing (at 1 day) the level of fat and moisture reflect the greater loss of fat from the aseptic vat manufacture. The aseptic vat cheeses were 3% higher in moisture and 3.5% lower in fat and, while the solids-not fat (SNF) difference was small, the difference in moisture in the non-fat substance (MNFS) and the fat in the dry-matter (FDM) reflected the differences in moisture and fat.

Table iv. The composition of milk, whey and 1 day cheeses made in the aseptic vats and the open vats.

	Milk		Whey		Cheese	
	AV[1]	OV	AV	OV	AV	OV
Volume (L)	85	350	67	285	-[2]	-
Total solids (%)	12.33	12.22	6.42	6.10	-	-
Moisture (%)	-	-	-	-	36.60	33.80
Fat (%)	4.15	4.01	.65	.34	33.50	37.00
pH	6.59	6.61	5.93	5.93	5.03	5.08
Casein (%)	2.49	2.49	-	-	-	-
Total Nitrogen (mM/kg)	-	-	.127	.139	-	-
Solids not fat (%)	-	-	-	-	29.90	29.20
Calcium (mM/kg)	30.2	31.1	13.20	13.60	174	173
Phosphate (mM/kg)	-	-	11.70	11.70	-	-
Salt (%)	-	-	-	-	1.70	1.55
Salt in moisture (%)	-	-	-	-	4.64	4.59
Ca/SNF (mM/kg)	-	-	-	-	582	592
MNFS (%)	-	-	-	-	55.10	53.60
FDM (%)	-	-	-	-	52.90	55.90

[1] AV = Aseptic vat, OV = Open vat
[2] - = not determined

The differences in composition between the two cheeses appear to be due almost entirely to a greater loss in fat from the aseptic vats and consequently, in comparative experiments, efforts were made to ensure that the milk used in the aseptic vats was of a higher fat content. It was also encumbent on the operator to ensure that all possible care was taken to reduce curd damage (and therefore additional fat loss) particularly at the initial stages of syneresis and agitation.

I.A.1.11 Efficiency of the Design. In order to establish the efficiency of the vat design it was necessary to answer two questions: i) could cheese be manufactured to an acceptable composition? and ii) were contaminating organisms excluded?

The results of comparative trials (Section I.A.1.10), indicated that the composition of the cheeses was within the bounds of normal Cheddar cheese, although the vats caused a higher than normal loss of fat. This loss of fat meant that the manufacture of aseptic vat cheeses required particular care to maximise the fat content of the milk, to minimize the fat losses, and to reduce the moisture of the curd.

The ability of the vats to exclude contaminating bacteria was examined in Section I.A.1.5. The most successful previous designs for large vats (Chapman et al., 1966; Kleter, 1975) both involved the autoclaving of the vats. However swabbing of the vats and the incubation of UHT milk indicated that no NSLAB were resident in the vats even though they were not sterile. Thus the vats should be adequate for the manufacture of NSLAB-free cheese, however the manufacture of starter-free cheese would probably not be possible, since the growth of starter in the milk helps to prevent the growth of undesirable contaminating organisms.

I.A.2 Manufacture of "NSLAB-free" Cheeses from Non-Aseptically Drawn Milk.

I.A.2.1 Background. Milk, as secreted, does not contain NSLAB (Kleter, 1974), however, by the time the milk is received by the processing plant, levels of NSLAB in the range of 100 - 1000 colony-forming units (cfu)/mL have been reported (Naylor and Sharpe, 1958; Perry and Sharpe, 1960; Sharpe and Mattick, 1960; Franklin and Sharpe, 1963). Since it has apparently been possible to manufacture NSLAB-free cheese from pasteurized, bulk milk (Fryer, 1968; Lowrie et al, 1974; Law et al, 1976) it was decided to manufacture cheese aseptically from pasteurized bulk milk.

I.A.2.2 Experimental Approach. Eight vats of cheese were manufactured in the aseptic vats during April and May 1984, from milk collected from the bulk storage vat of one town-supply dairy farm. The combined night plus morning's milk was run from the storage vat into steamed cream cans immediately after the completion of the morning milking.

The eight vats of cheese comprised four replicates with the cheese from one vat of each replicate being matured at 10 C and the other at 15 C. Each cheese was analysed for chemical composition at 1 day, and for bacterial count at 1, 14 and 35 days and at 2, 3, 6 and 9 months.

These cheese also formed the control cheese for a trial (Section II.3) where various NSLAB cultures were deliberately added to the aseptically manufactured curd at milling. In all, milk was collected on 13 occasions.

I.A.2.3 Milk Composition. The raw and pasteurized milk counts of the various milks are shown in Table v. The mean values were calculated using the logarithm of the numeric count. The mean total count of the raw milks was $1.24*10^4$ cfu/mL and of the pasteurized milks was $7.5*10^2$ cfu/mL. The mean NSLAB count in the raw milks was 377 cfu/mL. While a mean NSLAB count on the pasteurized milks would be meaningless since log1=0, counts of less than 1 cfu NSLAB in each of 5, 50 mL samples were recorded on only 4 of the 13 days. When isolates of the surviving NSLAB were identified, the most common type was a heterolactic coccus-like organism which produced D-lactic acid in milk and appeared to be similar to *Leuconostoc lactis*.

I.A.2.4 Cheese Composition. The mean data for the cheese composition obtained from the analysis of variance is presented in Table vi. The compositional factors were all within the bounds of normally accepted Cheddar cheese in N.Z. except that the moisture (and hence the MNFS) were slightly higher than normal. This is a feature of the aseptically manufactured cheeses and would be expected to result in cheeses which matured more rapidly.

Table v. Bacterial counts in 13 raw milks collected from the bulk vat of one town-supply dairy farm, and in the same milks after pasteurization.

	Raw Milk			Pasteurized Milk						
	Coli[1] (/mL)	Total (*10^{-3}) (cfu/mL)	NSLAB (/mL)	Coli (/10mL)	Total (*10^{-2}) (cfu/mL)	NSLAB (/10mL)				
1	90	7.3	130	<1	6.5	<1	1	1	<1	2
				<1	47.0					
2	720	2.2	390	<1	16.0	<1	1	<1	<1	<1
				<1	6.0					
3	270	40.0	4000	<1	42.0	<1	1	<1	86	4
				<1	79.0					
4	90	18.0	420	<1	9.0	<1	<1	<1	6	<1
				<1	8.0					
5	3600	13.2	1300	<1	3.3	<1	<1	5	<1	4
				<1	3.0					
6	2400	12.0	140	2	17.0	<1	13	<1	<1	<1
				<1	19.0					
7	200	4.2	44	<1	74.0	<1	<1	<1	<1	<1
				<1	4.7					
8	900	7.8	380	<1	3.6	<1	<1	<1	<1	<1
				<1	3.7					
9	1200	9.8	430	<1	3.5	<1	<1	<1	<1	<1
				<1	2.8					
10	1200	1.9	-	<1	3.8	<1	<1	<1	<1	<1
				<1	4.0					
11	740	14.0	-	<1	2.3	<1	1	1	<1	<1
				<1	1.7					
12	900	107.0	530	<1	4.7	<1	1	<1	<1	<1
				<1	4.0					
13	240	93.0	-	<1	5.4	<1	<1	<1	10	<1
				<1	9.3					
Mean	562	12.4	377		7.5					
+SD	1806	42.7	1314		22.0					
-SD	175	3.6	109		2.6					

[1] Coli = count on VRB agar, Total = Count on SM agar, NSLAB = Count on LBS agar

I.A.2.5 Bacterial Counts in the Cheeses. The log mean counts of NSLAB in the cheeses (as computed by the analysis of variance) are presented in Fig 8. The NSLAB count in cheeses matured at 15 C rose to a plateau of $1*10^4$ cfu/g between 2 and 3 months and then continued to rise at a slower rate to reach $3*10^7$ cfu/g by 9 months, while at 10 C the NSLAB count rose relatively slowly to reach a plateau of $1*10^5$ cfu/g by 6 months.

Table vi. Mean 1 day composition of 15 cheeses made in the aseptic vats from non-aseptically drawn milk.

Moisture (%)	36.72
Fat (%)	33.72
Calcium (mM/kg)	180
Salt (%)	2.10
pH	5.19
MNFS (%)	55.2
FDM (%)	53.3
SNF (%)	31.5
S/M (%)	5.72
Ca/SNF (mM/kg)	571

I.A.2.6 Conclusions. The raw milk NSLAB counts were similar to those reported previously (Naylor and Sharpe, 1958; Perry and Sharpe, 1960; Sharpe and Mattick, 1960; Franklin and Sharpe, 1963). Since all were apparently not killed by pasteurization and grew in the control cheese during

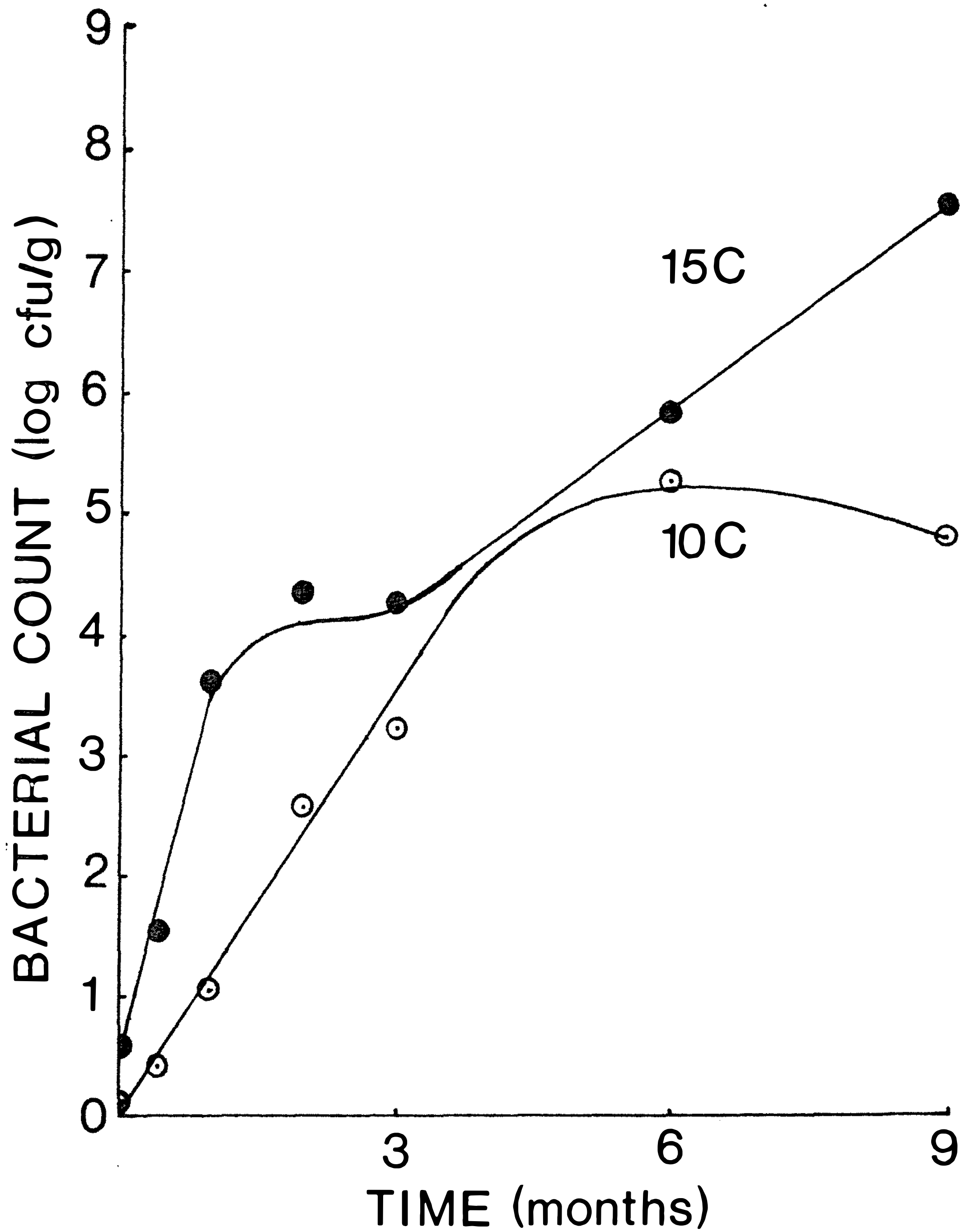


Figure 8. Growth of NSLAB organisms in control cheeses made in the aseptic vats from non-aseptically drawn milk. Cheeses were made in the aseptic vats from pasteurized milk obtained from the bulk tank of one dairy farm. The count of NSLAB organisms in the cheeses was determined over the 9 month maturing time at either 10 C or 15 C. ⊙ - ⊙ cheese matured at 10 C; ● - ● cheese matured at 15 C.

maturation, a project on their destruction by heat was commenced (Section I.A.3) leading to the use of aseptically drawn milk for cheesemaking (Section I.A.4).

I.A.3 A Microbiological Specification For Milk For Aseptic Cheesemaking.

I.A.3.1 Background. By the time milk is received by the processing plant, the level of NSLAB is normally in the range of 100 - 1000 cfu/mL (Naylor and Sharpe, 1958; Perry and Sharpe, 1960; Sharpe and Mattick, 1960; Franklin and Sharpe, 1963). In order to obtain NSLAB-free milk for the manufacture of NSLAB-free cheese, either the NSLAB must be prevented from entering the milk or the NSLAB must be removed.

A number of investigators have studied the destruction of NSLAB by heat. Evidence has been obtained that very small percentages of some NSLAB cultures can survive typical commercial pasteurization treatments (Slater and Halvorson, 1947; Perry and Sharpe, 1959; Franklin and Sharpe, 1963; Franklin, 1965; Fryer, 1968). Despite this evidence of thermal stability, some investigators have apparently been able to manufacture cheese aseptically from pasteurized bulk milk and find no NSLAB contamination (Lowrie et al, 1974; Law et al, 1976). Other investigators have reported occasional contamination using similar experimental conditions. In contrast, to manufacture NSLAB-free Gouda cheese, Kleter and Vries (1974) needed pasteurized milk aseptically drawn from selected cows.

Clearly there are uncertainties concerning the conditions that must be met in order to produce NSLAB-free milk for aseptic cheese manufacture. For this reason, and following a

period where organisms identified as Leuconostoc lactis were isolated from the pasteurized milk, a study was undertaken to determine the heating conditions required to kill NSLAB and thereby establish whether it is necessary to use aseptically drawn milk to manufacture NSLAB-free cheese.

I.A.3.2 Methods.

I.A.3.2.1 Cultures. Pasteurization experiments were conducted using pediococci, leuconostocs and lactobacilli inoculated into sterile chilled reconstituted skim milk (RSM). Prior to each experiment a working culture was grown for 24 h at 30 C in MRS broth (Man, Rogosa and Sharpe liquid medium, Man et al, 1960) and 0.1 mL was inoculated into 10 mL of Yeast Glucose Litmus Milk (YGLM, Wheater, 1955) which was then grown at 30 C until clotted (24 - 48 h). One mL of the clotted culture was inoculated into 100 mL of YGLM which was incubated at 30C for 40 - 48 h. However, if the culture clotted YGLM in 24 h, it was employed at that time.

The YGLM culture was used to inoculate 3 L of autoclaved, reconstituted skim milk (3% inoculum) which had been chilled to 4 C. The inoculated milk was held at 4 C during the course of the experiment (< 4 h).

I.A.2.2 Pasteurizer. Three hundred mL volumes of chilled culture were pasteurized at various temperatures and for various holding times in a plate pasteurizer (Franklin, 1965). The operating conditions were similar to those employed by Franklin except that the plate was cleaned each day by soaking with alkali and was sterilized by overnight

treatment with Iodophor. Sterile RSM samples were poured into the plate at the begining and end of each run to check for contamination.

I.A.3.2.3 Plate Counting. The bacterial count of samples before or after heating was determined after serial dilution of the sample in Ringers diluent and pour plating with MRS Agar (Man *et al*, 1960).

Plates were incubated aerobically at 30 C for 36 - 72 h, depending on strain requirements. For *L.casei* NCDO 161, heated cultures gave variable colony sizes on MRS agar. The omission of citrate combined with anaerobic incubation improved the recovery of injured cells. However this modification was unnecessary with the other strains.

I.A.3.3 Results.

I.A.3.3.1 Target Level for the Destruction of NSLAB. With any system of bacterial destruction (such as pasteurization) there is a statistical probability that some individuals may survive. It is therefore important to define the maximum tolerable level of survival. For the purposes of the present study, this was 1 per 10 kg of cheese, as even if every survivor grew to 10^8 individuals (i.e. 10^4 cfu/g cheese), each colony would be fixed within the block and the likelihood that it would influence the flavour and texture of the greater mass of the whole cheese would be negligable. Since Cheddar

cheese curds are concentrated by a factor of about 10 during whey removal, a level of 1 NSLAB per 100 kg of pasteurized milk is equivalent to 1 NSLAB per 10 kg of cheese.

Levels of NSLAB in good quality raw milk have been reported around 1000 cfu/mL (Naylor and Sharpe, 1958; Perry and Sharpe, 1960; Sharpe and Mattick, 1960; Franklin and Sharpe, 1963), therefore, a target destruction level of 10^8 would be required to produce "NSLAB-free" pasteurized milk from bulk raw milk.

I.A.3.3.2 Survival Following Heating for 15 s. The effect of heating at various temperatures for 15 s (the minimum holding time used in commercial pasteurization) was used as a screening test for thermal sensitivity. The effect on the count of *L. plantarum* 8014 is illustrated in Fig 9. There was little decrease in count below 58 C, and 10^8 reduction in count was obtained by 68 C. The data for 21 other cultures are shown in Table vii. At 72 C, 15 s was sufficient to achieve at least a 10^8 reduction in count for 13 cultures, comprising all the leuconostocs except *Leu. lactis*, and some pediococci and lactobacilli. However, 8 strains had less than 10^8 reduction, with the least sensitive (*L. casei* NCDO 161) only reduced by $10^{3.5}$. These values compare with reported levels of destruction during pasteurization of 10^4 to 10^6 by Franklin (1965) using Pediococcus 989 and by Fryer (1968) using an *L. casei* var. *casei* isolate.

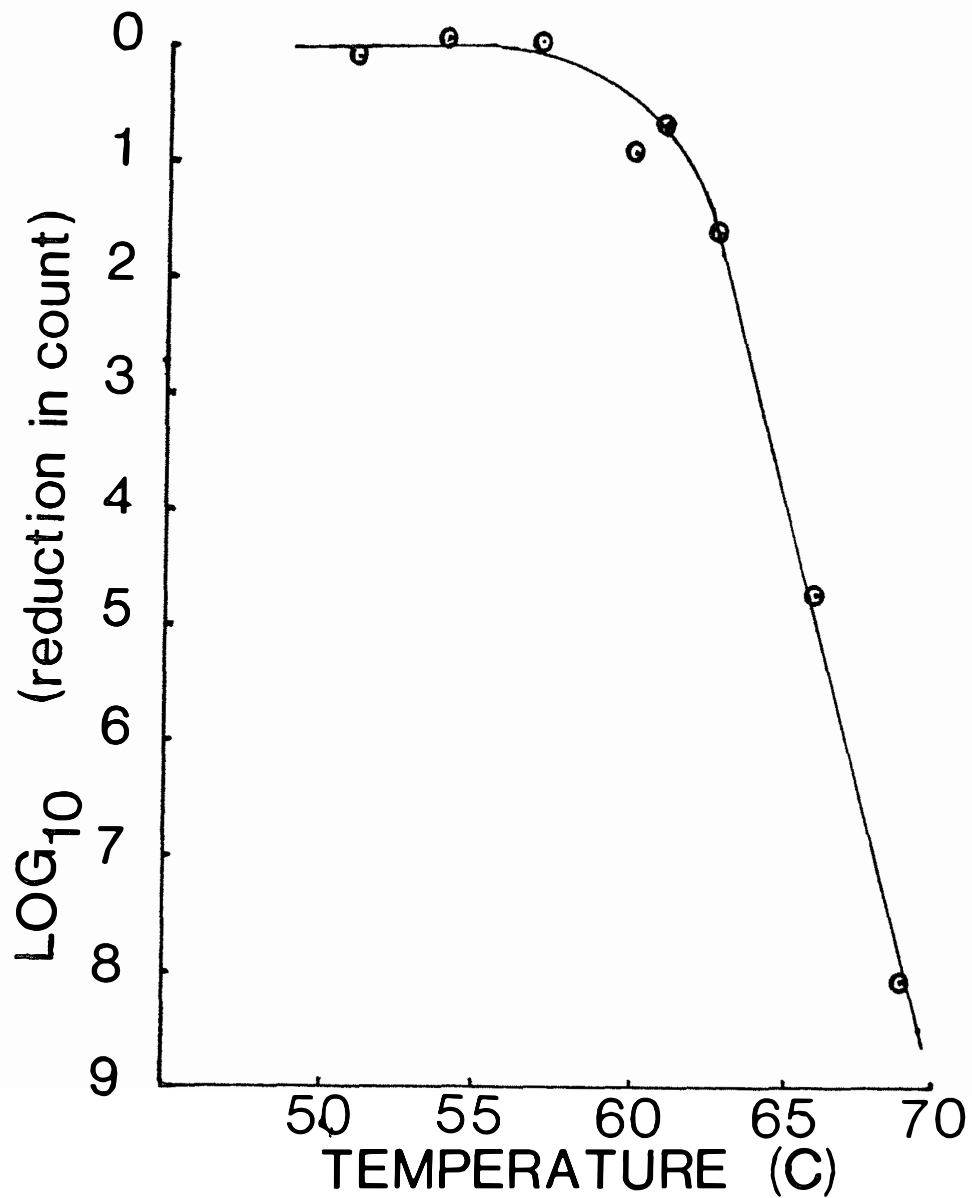


Figure 9. Thermal death of L. plantarum 8014. Sterile reconstituted skim milk at 4 C was inoculated with culture and then 300 mL aliquots were heated at various temperatures and held for a constant time of 15 secs in a laboratory plate pasteurizer.

Table vii. Thermal death of NSLAB cultures inoculated into RSM and held for 15 s at temperatures between 50 and 80 C.

Culture Name	Designation	Number of logs reduction in count in 15 s at 72 C	Culture temperature at 15 s for 8 logs reduction in count
Leu mesenteroides	NCDO 523	15	65
Leu paramesenteroides	NCDO 803	16	66
Leu dextranicum	NCDO 529	14	66
Leu dextranicum	NCDO 812	14	66
Pediococcus pentosaceus	NCDO 990	12	69
L. plantarum	ATCC 8014	12	68
Pediococcus pentosaceus	NCDO 1850	11.5	69
Homolactic lactobacillus	38	10.5	68
Leu cremoris	NCDO 543	9	70
L. fermentum	ATCC 9338	9	72
Homolactic lactobacillus	45	9	72
L.brevis	NCDO 1749	8	72
L. casei var. *rhamnosus*	ATCC 7469	8	72
Homolactic lactobacillus	43	7.5	72
L. fermentum	ATCC 6991	6.5	74
Heterolactic lactobacillus	2	6.5	74
Leu lactis	NCDO 533	6	75
pediococcus	39	6.5	75
Pediococcus pentosaceus	NCDO 1220	5.8	75
Homolactic lactobacillus	44	7	75
L. casei var. *casei*	NCDO 161	3.5	83[1]

[1] Recovered anaerobically on MRS agar without citrate, since thermally injured cells of this strain (unlike the others) were inhibited by the high citrate levels in standard MRS.

I.A.3.3.3 Thermal Death Kinetics of Selected Strains. Death kinetics are usually characterized as the D value - the time taken to reduce the count of a culture 10 fold at a specified temperature (Davis *et al*, 1968) - and the Z value - the change in temperature required to increase the D value 10 fold (Slater and Halvorson, 1947; Ingram, 1969). Two cultures, with different thermal sensitivities, were selected for more detailed examination. The reduction in count was measured at

different holding times and temperatures. The resultant D plots (i.e. log count versus time at fixed temperature) were linear. Z plots (i.e. log D versus temperature) were non-linear and indicated a reduction in the rate of kill with increasing temperature (Fig 10). Z-values were obtained from the slopes of the Z-plots. For L.plantarum 8014, the curve gave a Z-value at 54 C of 5.0 C while at 68 C the value had risen to 9.0 C. For L. casei, the Z-value at 55 C was 2.4 C rising to 11 C at 75 C.

Non-linear Z-plots are well known with vegetative cells (Brown and Melling, 1971; Moats, 1971; Moats et al, 1971), and curves are postulated to be due to a mixed population. In the present work, any mixture of sensitivities could not be genotypic since for both strains, single colony isolates of high temperature survivors were no more sensitive than the original culture (data not shown).

I.A.3.4 Conditions Required to Obtain "NSLAB-free" Milk. The least sensitive NSLAB strain tested (L. casei 161) would require temperatures in excess of 83 C for 15 s to achieve a 10^8 reduction in count. Calculating from the D-value at 72 C, a holding time in excess of 58 s would be required to achieve the same reduction in count. Both these conditions far exceed the thermal treatments used in commercial practice and introduce the possibility of protein denaturation which would be detrimental to coagulation and syneresis during cheesemaking. Since there are NSLAB which survive

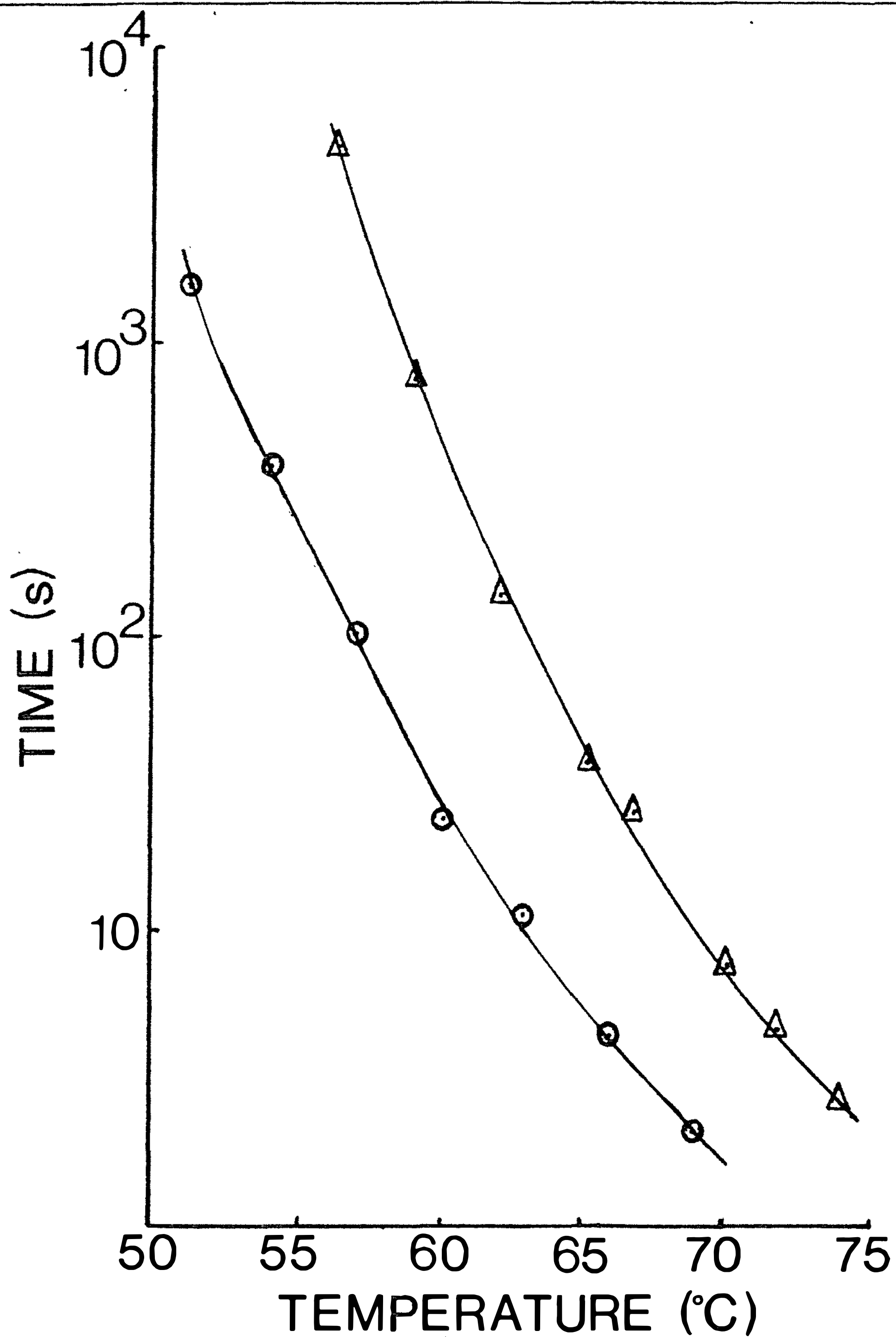


Figure 10. Z-values of two representative NSLAB cultures. Two cultures, representative of the range of thermal sensitivities were selected. The D values (time in secs for a decrease in count of 1 log i.e. 90%) were determined at various temperatures and these data were plotted to determine the Z values (temperature change required to change the D value by 1 log i.e. 90%): ⊙-⊙ , L.plantarum 8014; △-△ L.casei NCDO 161.

pasteurization, aseptic milking of the cows was used when obtaining milk for all furthur experiments with the aseptic vats. The methods for aseptic milking and the proof that milk was NSLAB-free are reported in Section I.A.4.

I.A.4 Aseptic Milking and the Proof of NSLAB-free Conditions.

Since cheeses made from milk collected from the farm vat became contaminated with NSLAB (1.A.2) and NSLAB can survive pasteurization (1.A.3), all further cheeses were made from milk drawn aseptically. This chapter describes the methods for obtaining aseptic milk and presents the bacterial counts found in those milks and in cheese-like "slurries" made from them.

I.A.4.1 Aseptic Milking Procedures.

I.A.4.1.1 Methods.

Cows. Milk was obtained aseptically from cows of the Dairy Cattle Research Unit, one of the seasonal supply herds of Massey University. Between 8 and 12 cows were milked for each cheesemaking trial, the number depending on the volume of milk being given at that stage of lactation. Two sequential collections were made, on the night preceding the day of manufacture and on the morning of that day.

Equipment. The milking equipment consisted of two complete sets of milking cups and test buckets. The claw pieces were stainless steel to enable autoclaving and the rubberware was heat tolerant butyl rubber (Reid Milkerware, Reidrubber Ltd., Auckland, N.Z.).

Sterilization. The assembled cups and rubber tubing were wrapped in Kraft paper. The lids of the test buckets were secured with autoclave tape and the tube fittings filled with cottonwool and covered with microbiological tube closures. All were then autoclaved at low pressures (7-9 psi, >110 C) for 30 min. Higher pressures were avoided to prevent excessive deterioration of the rubberware. The milking equipment was assembled and each unit attached to the plant in place of one of the normal milking cups.

Preparation and Milking of the Cows. The cows were prepared following the method of Kleter and Vries (1974) except that there was no preliminary microbiological screening of the cows prior to milking. All the cows were kept as part of the milking herd except that, during milking of the herd, those selected for aseptic milking (identified with coloured collars) were separated into a side yard as they presented themselves at the bails. After milking of the herd was completed and the equipment cleaned, the "aseptic group" were brought to the bails and milked.

The cows were milked in four bails of an internal race walk-through shed. While two animals were being milked, the two beside them were being prepared. The udders were first washed with warm untreated tap water to soften and remove gross contaminating material. They were then further washed with iodophor treated water to remove any last traces of dirt and begin sterilization of the skin. Particular attention was paid to the sides and tips of each teat. Finally, after

approximately three minutes, the teats were wiped using paper towelettes soaked with 95% industrial alcohol. This served to dry the teats and provide further sterilization. Wherever possible, the milking cups were attached within one to two minutes of the alcohol treatment.

The cups were attached to each sterilized udder in turn. No attempt was made to resterilize the cups between each cow. The maximum number of cows milked through each set of cups at each milking was seven.

Between individuals (as required) the test buckets were emptied by pouring the milk into presterilized (121 C/15 min) 45 l aluminium cream cans fitted with overhanging lids. Before milk transfer, the lips of the test buckets were sterilized by wiping with alcohol.

Milk collected in the evening was chilled at 4 C and held overnight at that temperature by immersion of the cans in thermostatically controlled chilled water baths. Milk collected in the morning was not chilled before use.

I.A.4.1.2 Bacterial Count in Aseptically Drawn Milk. The mean bacterial counts of all 48 raw, aseptically drawn milks taken over three seasons, are shown in Table viii. The raw milk total count averaged (log mean) $5.26*10^3$ cfu/mL and the count of micrococci and staphylococci averaged $1.68*10^3$ cfu/mL. Low numbers of coliform organisms were detected, averaging 44 cfu/10 mL and on 17 occasions some organisms grew

Table viii. Mean bacterial counts from 45 aseptically drawn raw milks collected over three manufacturing seasons.

		Agar	Log mean count	+s.d.[1]	-s.d.
Raw Milks					
Coliforms	(cfu/10mL)	(VRBA)[2]	0.75	5.28	0.11
Total Count	(cfu/mL)	(SMA)	$5.26*10^3$	21.6	1.28
Micrococcus	(cfu/mL)	(BP)	$1.68*10^3$	3.58	0.73
NSLAB	(cfu/10mL)	(LBS)	0.14	0.27	0.07
Pasteurized Milks					
Coliforms	(cfu/10mL)	(VRBA)	Absent (<1)	N/A[3]	N/A[3]
Total Count	(cfu/mL)	(SMA)	4.11	44.2	0.38
Micrococcus	(cfu/mL)	(BP)	0.15	0.39	0.06
NSLAB		(LBS)	<1 in all 5, 10 mL samples on 46 of the 48 days.		

[1] s.d. = standard deviation
[2] N/A = not applicable. Since there were no coliforms present, statistical analysis was impossible.
[3] VRBA = violet red bile agar; SMA = standard methods agar; BP = Baird Parker agar; LBS = lactobacillus selection agar.

on LBS agar. These were contaminants growing with the presence of the milk solids in the agar and did not grow in MRS broth at 30 C when isolated.

After pasteurization at 72 C for 15 s, the log mean of the total and micrococcus counts in the vat milks had dropped to 4.1 cfu/10 mL and 1.5 cfu/10 mL respectively. No coliform organisms were detected in 10 mL of vat milk and on only two occasions were any NSLAB organisms found in 5, 10 mL samples of pasteurized milk.

I.A.4.2 Bacterial Counts in "Slurries" Made from Aseptically Drawn Milk.

In order to determine whether NSLAB existed in the aseptically drawn milks at such low levels as to be unrecoverable by direct plating on LBS agar, a number of trials were conducted where "slurries" were made directly from the aseptic milk and incubated at 30 C. The method developed was a modification of the slurry method of Kristoffersen *et al.*, 1967. Whereas Kristoffersen made slurries to 40% total solids from pressed cheese curd on the day after manufacture, this new method avoided the cheese vat completely in order to determine the NSLAB loading of the milk alone.

I.A.4.2.1 "Slurry" Manufacture. "Slurries" were made in presterilized (121 C/15 min) 1 litre polycarbonate bottles (Nalgene, Cole Parmer Instrument Co., Chicago, Ill., USA) held in a temperature controlled water-bath, from 1 L of milk (either raw aseptic; pasteurized (62.5 C/30 min) aseptic; or pasteurized (72 C/15 s) factory milk). To 1 L of milk at 32 C was added 21 mL of starter (1:2 by volume of coagulated cultures of *S. cremoris* 584 and *S. cremoris* 134) followed by 0.16 mL of rennet (N.Z. Standard strength). After 40 min for coagulation, the curd was cut with a presterilized (121 C/15 min) spatula into columns approximately 10 mm square. The jar was shaken after closing the lid. The curds and whey were cooked to 38 C over 40 min using a temperature controller of

the same design as those on the aseptic vats, attached to the water-bath heater. When 38 C had been reached, 400 mL of whey was poured off and 250 mL of sterile water added to reduce the lactose content of the curd. The curd and whey was then held until 2 h 50 min after starter addition with slow cooling to 36 C. During cooking and holding, the jar was shaken approximately every 15 min to ensure that the curd did not mat on the bottom.

At 2 h 50 min after starter addition, the whey was removed ("running") and the curd allowed to "cheddar" on the bottom of the jar. At approximately 15 min intervals, the curd block was gently inverted in the bottom of the jar, and any surplus whey was removed. Only enough whey was removed to reduce the moisture content of the final "slurry" to 60%.

One hour and 50 min after running, the curd was cut with a spatula and moved from the jar into a 250mm * 200mm cheese bag (C20, film 9502, Wrightcel, Feilding, N.Z.). Salt (autoclaved at 121 C for 15 min) was added to a salt-in-moisture content of 5% (approx. 5.4 g), and the contents thoroughly mixed. The salted curd was then divided between 5 cheese bags which were sealed under vacuum and incubated at 30 C in an air incubator. Each day the bags were squeezed to mix the contents, and the contents of a separate bag were analysed at each sampling time.

I.A.4.2.2 Bacterial Counts in the "Slurries". The results of one of a number of trials involving the manufacture of 40% solids "slurries" from aseptically drawn milks are shown in Fig 11. In the "slurry" made from the raw aseptically drawn milk, neither NSLAB nor coliform organisms were detected even after 10 days at 30 C. The count of micrococci rapidly increased to levels around 10^7 cfu/g at 1 day and plateaued at around 10^6 cfu/g. The apparent count of starter organisms (total count on M17 agar, Terzaghi and Sandine, 1975) rapidly dropped in the first few hours and then more slowly until reaching levels similar to the micrococci.

In slurries made from aseptically drawn milk pasteurized at 62 C for 30 min, no coliform or NSLAB organisms were detected and the growth of the micrococci was delayed due to their initially lower count. The count of starter organisms decreased to lower levels than in the raw milk slurries reaching levels of 10^4 cfu/g by 10 days.

For comparison, milk from normal factory supply, and pasteurized in a commercial pasteurizer was also made into slurry using the same aseptic techniques. While no coliform organisms developed, the count of NSLAB increased steadily throughout the incubation period, reaching levels of 10^7 cfu/g by 10 days. The micrococcus count, while initially at least 10 times higher than in the pasteurized aseptically drawn milk, did not rise to more than 10 cfu/g through the incubation period. The starter count was similar to that found in the slurry from aseptically drawn milk.

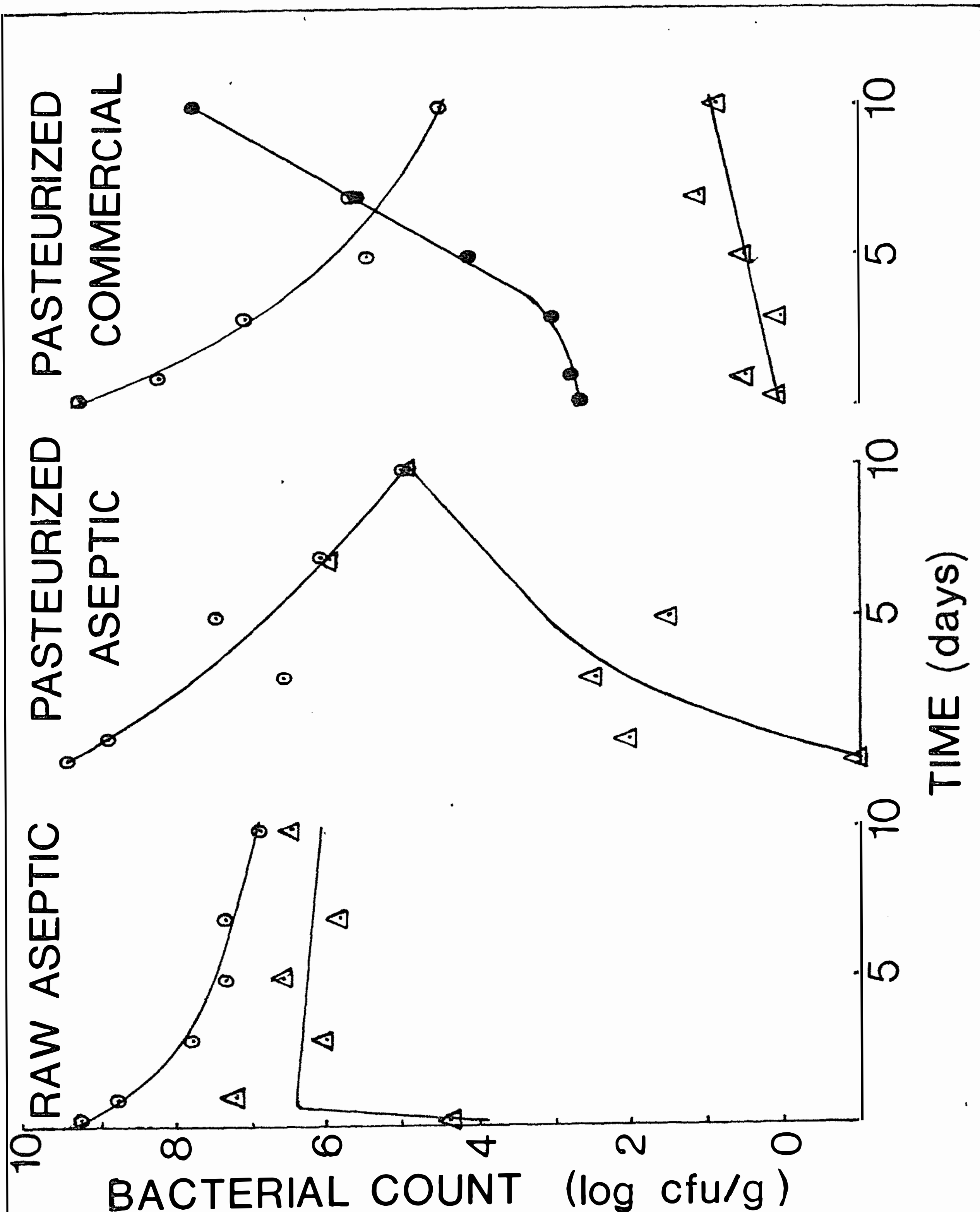


Figure 11. Counts of starter, NSLAB and micrococcus organisms in "slurries" made under strict aseptic laboratory conditions. Bacterial counts were determined in "slurries" made from either raw or pasteurized (62.5 C/ 30 min) aseptically drawn milk and from bulk, commercial raw milk HTST pasteurized in the Institute's processing hall. ⊙-⊙ starter count; Δ-Δ micrococcus count; ●-● NSLAB count.

I.A.4.3 Conclusions. To achieve the target of less than 1 NSLAB organism per 10 kg of cheese curd (1.A.3.3.1), allowing for a maximum survival of 10^{-3} on pasteurization, requires the absence of NSLAB in 100 mL of raw milk. The absence of NSLAB from "slurries" made from 1 L of unpasteurized raw milk showed that the milk, drawn with the relatively simple precautions described, adequately met the NSLAB specification. However, if the experimental aim were to produce starter-free cheese, it would be necessary to revert to the more stringent precautions used by Kleter and Vries (1974).

The absence of NSLAB from the "slurries" made from aseptically drawn milk also showed that the various additives required during the cheese-making process were also free from NSLAB contamination. These included the starter culture (absent in 21 mL), rennet (absent in 16 mL), salt (absent in 5 g) and cheese bags (absent in 0.05 m^2 of internal surface area).

I.B. Methods for Texture and Flavour Analysis of the Cheeses.

The texture and flavour of the cheese made during this project was assessed using a panel selected, trained and maintained for this project. The panel was organised by the staff of the Product Use and Evaluation Section, NZDRI, under the guidance of Dr H.R.Cooper.

I.B.1 Introduction. The assessment of cheese quality in terms of its texture and flavour has traditionally been the preserve of certificated graders with long experience. However, at least in Australia and New Zealand where cheese has traditionally been graded at a young age prior to export, it has been recognised that "traditional" organoleptic grading does not accurately predict the final flavour (Loftus-Hill and Silcock, 1970; Scott, 1974; Marshall and Waugh, 1978; McBride, 1979). Indeed it has been recognised that traditional flavour grading is mainly to "ensure that the cheese is free from objectionable flavours" (Pearce and Gilles, 1979). While this form of assessment is quite suitable for the screening of export products, cheese researchers have recognised the need to develop procedures that identify different flavours and to quantify their intensities (Morris, 1978).

Attribute identificaton and quantification has been a developing preoccupation among Food Technologists working with a range of products. This has developed as the limitations of preference-type hedonic scaling methods have become apparent. These limitations are particularly obvious when attempting to predict the acceptability of products to customers with different ethnic origins and perceptions. Thus the concept of flavour profiling developed, where different components of the total flavour were identified and recorded. Some objectivity was gained by category scaling, where the taster used words or numbers to describe the attributes of the product.

The flavour profile was developed by Cairncross and Sjostrom (1950) who sought a method to reliably detect small differences in food flavours when sodium monoglutamate was added. The common procedures of the time (mainly preference-type) were woefully inadequate since the difficulty in interpretation meant that small differences in flavour could not be reliably detected. The flavour profile method attempts to isolate components of the total flavour of the product, and to assign a value to the intensity of each identified component. Sjostrom et al (1957) in reviewing their publications on the subject, pointed out the essential feature of the profile method, that the panelists are trained to analyse the flavour components of the product. They do not attempt to express a preference or judgement on the product. The interpretation of the results as to which samples are "better" is a separate function.

The most advanced version of flavour profiling methods is currently the Quantitative Descriptive Analysis (QDA) system (Stone _et al_, 1974). The most important features of this profiling system are the use of a 6 inch anchored line for measuring the attribute intensities, and the collection of multiple judgements in a controlled environment. While the panelist training and sample preparation methods used in this thesis have borrowed heavily from the QDA system, the preference of the panelists for the use of a numerical scoring system (Section I.B.5) means that the method used could most closely be described as a flavour profile method rather than a QDA method.

In this project, where the aim was to discover whether NSLAB contributed to the generation of flavour in Cheddar cheese, flavour profiling was used rather than the more traditional grading procedures, since it would be possible to obtain evidence on which specific attributes were enhanced. With this knowledge, it should be possible to indicate what types of biochemical reactions were being catalysed by the presence of NSLAB organisms.

I.B.2 Details of the Conditions for Sample Assessment.

Location. The trialists for training as panelists made their assessments in the individual panel booths in the Institutes panel room. Training was mainly as "round-table" sessions in

the round-table room gradually transferring to full blind assessment in the panel booths, while all the actual sample assessments were made in the individual booths.

Sample Handling. Samples were allocated a random 3-digit number on their arrival at the preparation room. Individual cubes of 15*15*15 mm were cut from the cheese or in the case of plugs, each plug was cut into lengths of 5 mm. These individual segments were placed back in their bags and tempered at 12 C for 1 h before tasting.

Immediately before the panel session, individual samples were placed into wax coated paper cups (Lily cups, Winstone Merchants, Auckland, NZ) bearing the ramdom sample number. The required cups were placed onto a tray along with a polystyrene cup of pure tap water and a plate with a slice of fresh white bread for the panelists to use at their discretion for cleansing the palate.

All assessments were made under plain white lighting, since sample colour was not highly variable.

I.B.3 Selection of the Panelists. Since not all individuals are equally sensitive to all the flavour attributes present in cheese, a large number of people were screened for their ability to detect the differences between various cheese samples. The technique of "reforming" cheese at the same composition as the original cheese was used to prepare samples with varying intensities of one of a number of attributes.

I.B.3.1 Manufacture of Reformed Cheese. Rindless Cheddar cheese of various ages was selected and the surface 10 mm removed. The cheese was then cut into fingers (approximately 30*30*200 mm) and minced through a worm mincer (EB20F, Crypto-Peerless, London, UK) with a 4 blade cutter and an extrusion plate with 5 mm diameter holes.

The required portions of cheese or additive were weighed into a Z-mixer (C&H type SV) and a vacuum of 300 mBar was drawn for 2 min prior to mixing. The cheese was mixed for 1 min at 100 rpm. Following a further 1 min under vacuum the mixer was opened and emptied into a hand-cranked sausage extruder and the mixed cheese extruded through a 13 mm diameter nozzle into clear sausage casings 300 mm long and 30 mm in diameter. The casings were tied off and put into a 4 C refrigerator until required for the panel.

I.B.3.2 Sample Preparation and Presentation. The sample sausage was opened and the edges removed to make cubes of 15 mm side which were placed into plastic whirl-top bags (Whirl-pak, Nasco, Fort Atkinson, Wis., USA).

I.B.3.3 Experimental Method. The trialists were required to make triangle test assessments of 16 sample sets containing three samples (two identical and one different) presented two at a time over 8 days. The sample pairings are shown in Table ix.

Each trialist sampled at least 14 of the 16 triangle sets. The 16 trialists with the highest correct scores were selected for training as the QDA panel.

Table ix. Samples of reformed cheeses used for panel screening.

a) Reformed Cheeses Made.

Code	Description
2M	2 month cheese
C33	33% strong cheese in 2 month cheese
C16	16% strong cheese in 2 month cheese
C8	8% strong cheese in 2 month cheese
T	Strong cheese (over 9 months old)
S	2 month cheese plus added salt (5g/kg)
Bl	2 month cheese plus bitter peptides (3g/kg)[1]
Bh	2 month cheese plus bitter peptides (10g/kg)
1M	1 month cheese
50	50% 6 month cheese in 1 month cheese
6M	6 month cheese

b) Triangle Test Combinations for Panel Screening.

	Triangle 1	Triangle 2
Day 1	2M - C33	C33 - C8
2	2M - C8	6M - 1M
3	2M - Bl	1M - 50
4	50 - 1M	S - 2M
5	1M - Bl	C33 - C16
6	C33 - Bh	1M - C16
7	C33 - 1M	Bh - Bl

[1] Bitter peptides = Bitter UHT milk freeze-dried and added as powder plus water to maintain the original ratio of moisture to non-fat solids.

I.B.4 Panel Training. Following selection, the chosen panelists participated in round-table training sessions sampling a variety of different cheeses, and particularly, sampling Cheddar cheeses with a wide range of compositions and at a wide range of ages. Hence there was a wide range of both texture and flavour.

For the round table sessions, each panelist assessed a sample and then the results were shared and discussed around the group with the panel coordinator, Dr. Cooper acting as Chairperson. The project leader attended most of the sessions but only as a resource person not as a director. It was decided that the panel should, as much as possible, come to their own decision as to the name, number and definition of the attributes both of texture and flavour of the cheese.

After much tasting and discussion, a list of attributes was defined, and the panelists proceeded to a scale comparison test under fully blind-tasting conditions.

I.B.5 Scale Comparison. Twelve cheese samples were divided into two groups of six, group 1 comprising three very mild cheese and three aged cheese giving a wide spread of intensity, and group 2 comprising 6 cheese with only two to 3 months maturity difference giving a small spread of intensity. Each group of cheese samples was sub-divided into two groups of four (i.e. two duplicate samples per group) and presented to the panel on two occasions. The panel was asked to score the texture and flavour attributes one day on a category scale and the following day on a line scale. In this manner there were 16 samples analysed by both scaling systems.

The data was analysed by twoway analysis of variance of the panelist and sample effects for the daily data, the data for duplicate samples and the overall data of all 16 samples. The panelist variation was in all cases either the same for both scaling systems or very slightly less for the category scale.

Similarly, for the attribute intensities, only very minor differences in favour of the scaling system were found. As there were only slight differences between the methods and the panel preferred to use a category scale, that method of scoring was utilized for all further analyses. This decision effectively produced a flavour profiling method rather than a QDA method where the responses are recorded on an anchored line.

The list of attributes was changed once during the project- to separate the attribute "Residual Mouthfeel" into two attributes "mealy/curdy" and "gritty/sandy". The final list of attributes and their definitions is given in Table x, and a copy of the score sheet is presented in Table xi.

I.B.6 Data Analysis. The individual tasters results were entered by staff of the Product Use and Evaluation Section into a computer (Vax-VMS, Digital Equipment Corporation, Marlboro, Mass., USA) for data analysis by Analysis of Variance (Anova). The Anova analyses of samples from the individual experiments at each sampling time were performed by the PUES staff particularly in order to monitor panel performance. The combined data for all sampling times was collated by the project leader using coded entry to the Anova programme.

Table x. Cheddar cheese panel attribute terminology.

To evaluate texture, take a bite out of the cheese sample approximately 15*15*15 mm. Then measure the following attributes:

Firmness: The amount of force required to take the first bite of cheese, assessed using the front teeth.
Rubberiness: The degree to which the cheese returns to its initial form after biting, assessed during the first two to three chews.
Crumbliness: The degree to which the cheese structure falls apart and breaks up during the initial two to three chews.
Smoothness: The smoothness of the cheese against the palate as it breaks down during mastication.
Stickiness: The stickiness of the cheese against the palate and around the teeth during mastication.
Bittiness: A residual mouthfeel which is described by the presence of particulate matter in the mouth.
a) mealy/curdy: The presence of soft, curd-like particulate matter which is perceived during final mastication.
b) gritty/sandy: The amount of hard, particulate matter perceived during final mastication of the cheese (often caused by the presence of calcium lactate in the cheese).

Then evaluate the cheese for the following flavour terms:

Acid/Sour. Acid: A "clean" flavour similar to that of a dilute solution of mineral acid, usually perceived at the back and sides of the tongue.
Sour: A "dirty" flavour often associated with fermented-type flavours perceived at the back and sides of the tongue but tending to linger in the mouth as an aftertaste.
Fruity/Fermented: Associated with products that have been fermented. In cheese, this group of characteristic flavours include flavours variously described as: yeasty, alcoholic, ethanol, fizzy, effervescent, tangy, fruity.
Sulphide: Group of characteristic flavours in cheese which may have the distinctive character of hydrogen sulphide or may variously be described as feedy, weedy, cabbagy, oniony etc. (possessing a note similar to that found in the sulphur-containing vegetables).
Sharpness: A "peppery" characteristic perceived on the tongue which tends to linger - often associated with the flavour of very mature Cheddar cheese.
Bitterness: One of the four basic tastes perceived at the back of the tongue, tending to linger on as an aftertaste. In cheese this is caused by the presence of bitter peptide compounds and is similar to the bitterness found in UHT milk after prolonged storage.

Table xi. Copy of the score sheet used by the panel for scoring the cheese attributes.

Name ________________ Date ________________

CHEDDAR CHEESE PANEL

In front of you are several samples of cheddar cheese. Please evaluate them for the following characteristics using a 0-10 scale where

0	=	Absent
2	=	Threshold
4	=	Weak
6	=	Moderate
8	=	Strong
10	=	Intense

Sample Nos.

	____	____	____	____
TEXTURE:				
Firmness (Soft -> Firm)	____	____	____	____
Rubberiness (Not rubbery -> Very rubbery)	____	____	____	____
Crumbliness (Not crumbly -> Very crumbly)	____	____	____	____
Smoothness (Not smooth -> Very smooth)	____	____	____	____
Stickiness (Not sticky -> Very sticky)	____	____	____	____
'Bittiness' (Not 'bitty' -> Very 'bitty')				
a. Mealy/curdy	____	____	____	____
b. Gritty/sandy	____	____	____	____
Other ____________	____	____	____	____
FLAVOUR:				
Acid/Sour	____	____	____	____
Fruity/Fermented	____	____	____	____
Sulphide	____	____	____	____
Sharpness	____	____	____	____
Bitterness	____	____	____	____
Other ________	____	____	____	____

COMMENTS:

PART II. THE ROLE OF NONSTARTER LACTIC ACID BACTERIA IN CHEDDAR CHEESE RIPENING

Following the design and commissioning of the manufacturing vats (Part I.A), it was possible to manufacture cheese without the presence of NSLAB. Part II describes the results of experiments comparing aseptically manufactured cheeses with cheeses containing NSLAB.

Section II.1 gives a review of the literature pertaining to the role of NSLAB in cheese ripening, while Section II.2 gives the specific methods utilized.

Section II.3.1 compares aseptically manufactured cheeses without NSLAB to cheeses adventitiously contaminated with NSLAB by manufacture in open vats. Since the surface of the aseptic vat cheeses became contaminated with NSLAB during maturation, Section II.3.2 describes the source of this contamination. Section II.4 describes a further comparison of aseptically manufactured cheeses and open vat cheeses but with the surface of the cheeses protected from contamination by the addition of a wax coating before bagging. In a futher embedded comparison in Section II.4, both aseptic and open vat cheeses were matured rinded and waxed to establish the effect of the bag on the course of flavour development.

Section II.5.1 describes the selection of NSLAB cultures for addition to aseptically manufactured cheeses, and trials to determine the best techniques for their introduction. Section II.5.2 compares aseptically made cheeses with aseptically made

cheese to which a homolactic lactobacillus or a pediococcus culture was added. Since all the control cheeses in these trials developed a low level of NSLAB contamination and the differences noted were at best very small, this work was not repeated with cheeses having the surface protected as in Section II.4.

The final discussion and conclusions from Parts A and B of this thesis are drawn in Section II.6

II.1 Literature Review.

II.1.1 Occurrence, Source and Growth of the NSLAB Flora. The starter bacteria added to milk during Cheddar cheesemaking rise from an initial level of around $2*10^7$ cfu/g to around $2*10^9$ cfu/g during manufacture. During maturation, the count slowly decreases at rates dependent on type (Reiter et al., 1967) and strain (Martley and Lawrence, 1972). In all cheese after manufacture there is a low level of NSLAB which grow as the cheese matures to become the dominant flora within 1 - 3 months (Davis, 1935; Sherwood, 1939a; Mabbitt and Zielinska, 1956; Naylor and Sharpe, 1958a). The maximum count appeared to differ between cheeses (Hill and Thornton, 1958; Perry and Sharpe, 1960) but to be stable over long periods within any one cheese (Johns and Cole, 1959). The early literature on the microflora (particularly the NSLAB) in cheese has been well reviewed (Fryer, 1969).

The NSLAB flora of cheese is composed of various strains of mesophilic lactobacilli, pediococci, and possibly also leuconostocs. Early reports considered only lactobacilli with passing reference to leuconostocs, especially in young cheese. Homolactic lactobacilli (L. plantarum and L. casei) dominated with varying reports as to which of these strains were more prevalent (Davis ,1935; Sherwood, 1939b; Naylor and Sharpe, 1958a; see also review by Marth, 1963). Pediococci have also been reported (Naylor and Sharpe, 1958b), even being the dominant flora (Fryer and Sharpe, 1966). While

heterolactic lactobacilli have often been isolated (Perry and Sharpe, 1960) they are not now believed to dominate the NSLAB flora at least in N.Z. cheese (Thomas, 1986). However they may form a significant population if the cheese has been held at high temperatures during early maturation (Fryer, 1982), or if present in the cheese at unusually high levels - for example if introduced in the rennet (Stathouders and Veringa, 1967; Stathouders, 1968).

For most practical purposes, the NSLAB can be defined as those bacteria which grow on LBS agar plates (Rogosa et al, 1951) when incubated for 5 days at 30 C, usually anaerobically. This definition will exclude most if not all leuconostoc organisims which do not grow well on LBS agar.

NSLAB organisms occur in raw milk, but most are destroyed by pasteurization (see Section I.A.3.3.2) and so only contribute to the cheese flora if present in the raw milk at high counts (Naylor and Sharpe, 1958c). Therefore the major source of NSLAB appears to be post pasteurization contamination from the manufacturing equipment (Sharpe and Mattick, 1960). Microscopic investigation of ripening cheese (Dean et al, 1959; Rammell, 1960) has shown the presence of bacterial clumps in crevices and at the interfaces of curd particles, consistent with the contamination of the curd during manufacture.

The rate of growth of NSLAB is dependent on the maturation temperature of the cheese particularly in the initial period of cooling after pressing (Fryer, 1982). Both the rate of

growth and the balance of species is believed to be affected. There is little direct evidence for the influence of other compositional variables on the rate of growth of NSLAB in cheese. Salt inhibits growth (Hunter, 1950) and may selectively inhibit some species more than others (e.g. selective inhibition of pediococci, Nunez, 1976). Hunter (1950) reported a requirement for various metal ions, particularly K and Mn, and suggested that cheese may be deficient in these ions. The pH optimum for growth of lactobacilli is reported to be between 6.0 and 7.5 (Davis and Thiel, 1939), well above the pH of maturing cheese. It has been reported (Lawrence et al., 1983) that there is a tendency for pediococci to dominate in low pH cheese (e.g. Cheddar) and lactobacilli in high pH cheese (e.g. Swiss).

Of particular interest for the growth of NSLAB in cheese is the stimulation of growth obtained by the addition of starter lysate (Hansen, 1941; Branen and Keenan, 1969). Recently, NSLAB have been shown to "cannibalize" starter bacteria (Thomas 1986b) however it is not yet clear whether this is the source of their growth requirements in cheese.

II.1.2 Metabolic Capability of NSLAB. The role of NSLAB (in particular heterofermentative lactobacilli) in the formation of various defects in cheese is well documented. Sherwood (1939a) reported the role of lactobacilli in the formation of slit-openness in Cheddar cheese, confirmed later by Hoglund et al (1972b). The formation of a related problem, tallowy discolouration was also traced to lactobacilli (Barnicoat,

1950). The formation of various flavour defects when lactobacilli were deliberately added to cheese has already been reported (see Introduction). However it is not clear whether NSLAB contribute to desirable cheese flavour. NSLAB may contribute to lactose fermentation in the event that the lactose is not removed by the starter. Even homofermentative strains may produce such potentially flavourful compounds as acetaldehyde and ethanol (Keenan and Lindsay, 1968). However, under normal conditions, all the lactose is removed before NSLAB counts rise to high levels (Turner and Thomas, 1980).

Various lactobacilli isolated from dairy products are capable of utilizing citrate in liquid media (Fryer, 1970). Since the citrate present in milk is not fermented by the *S. cremoris* starter bacteria normally used in Cheddar cheese manufacture in N.Z., it is likely to be present in the cheese. Bacterial fermentation of citrate is accompanied by the formation of CO_2 and acetate along with the diversion of pyruvate metabolism from lactate to compounds including, acetate, formate, ethanol, and diacetyl and acetoin (Hickey *et al*., 1983b). From the viewpoint of flavour production, the most important products have been believed to be acetate and diacetyl. In Cheddar cheese, acetate is the dominant "free fatty acid" (Evans and Mabbitt, 1974) believed to form the basis of Cheddar cheese flavour (Patton, 1963).

The conditions required for NSLAB to metabolize citrate in Cheddar cheese are unknown. All 7 strains of *L. casei* isolated by Fryer (1970) utilized citrate in the absence of

fermentable carbohydrate as did 9 of the 10 strains of L. plantarum. Keenan and Lindsay (1967) reported that L. casei produced significantly higher quantities of diacetyl from citrate in the absence of carbohydrate than did L. plantarum. This may be explained by the finding that acetoin and diacetyl production by L. plantarum required an energy source (El Gendy et al, 1983).

NSLAB organisms are also capable of aerobic metabolism of various substrates in the presence of oxygen, leading to the formation of products other than lactate from pyruvate (for review see Condon, 1983). This is of particular significance since the NSLAB flora in cheese, particularly lactobacilli, have been implicated in the maintenance of a low redox potential in cheese (Davis, 1932; Kristoffersen, 1967; Manning, 1979). It is believed that the starter bacteria become incapable of maintaining the low redox potential that they initially obtain in the curd. The lactobacilli then take over the role of maintaining the low potential required for the catalysis of the ripening reactions.

Both carbohydrates and lactate can be oxidatively metabolized, with the greatest rate of metabolism apparently occurring at the end of the growth phase (Sedewitz et al, 1984). Other substrates for oxidative metabolism by both lactobacilli and pediococci include citrate and peptides (Thomas, 1986, 1987a).

As proteolysis is considered to form a major part of the cheese ripening process, a further role of the NSLAB flora could be to produce further proteolytic enzymes in the cheese curd. The early work on the effect of lactobacilli on proteolysis in cheese ripening has been reviewed by Fryer (1969). More recent data indicates that at least some of the lactobacilli in the NSLAB flora may contain significantly higher proteolytic activities than the starter streptococci (Hickey et al, 1983a).

The final area in which the NSLAB flora may promote flavour development is the production of low molecular weight sulphur compounds which have been implicated in the mature flavour of Cheddar cheese (Kristoffersen, 1973). Lactobacilli have been reported to produce H_2S (Kristoffersen and Nelson, 1955; Sharpe and Franklin, 1962), although there appears to be little correlation between NSLAB count and flavour (Fryer, 1969) or H_2S concentration and flavour (Aston and Douglas, 1983).

II.1.3 Aims of This Part of the Project. Since NSLAB are present in cheese at levels high enough to cause significant changes to the cheese, and they have the capability to produce significant flavourful compounds and proteolytic enzymes, it seemed appropriate to reconsider the possibility that they influence the development of desirable flavours in the cheese. This was even more timely due to the significant changes in technology that have occurred in the past 20 years,

in particular, the development of highly impermeable cheesebags, and the tendancy to a sweeter make with a lower flavour profile in the mature cheese.

The primary aim was to compare the effect of NSLAB on the development of Cheddar cheese flavour. This was accomplished by the manufacture of cheese aseptically (without NSLAB contamination) and in open vats with adventitious NSLAB contamination. The results are reported in Section II.3.1. Since the surface became contaminated by NSLAB during maturing (Section II.3.2) these experiments were repeated and the cheese surface waxed before bagging (Section II.4).

The addition of either of two representative NSLAB cultures (an L. plantarum isolate and a P. pentosaceus isolate) under controlled conditions was attempted in order to investigate whether the addition of NSLAB added to the flavour or produced off-flavours (Section II.5). Since these trials were attempted before the surface contamination was realized, and the differences between cheeses with or without NSLAB were very small, these trials were not repeated with cheese surfaces waxed.

II.2. Methods.

II.2.1 Culture History and Maintenance.

Starters. The *Streptococcus cremoris* strains 584 and 134 were used as starter cultures for all cheese maufacture. The strains were obtained from the culture collection of the New Zealand Dairy Research Institute and stored frozen at -75 C (Revco Inc., West Columbia, SC, USA) partially grown in sterile reconstituted skim milk (RSM). At approximately monthly intervals a vial was thawed (at 30 C) and 0.2 mL of culture was inoculated into 10 mL of sterile RSM. This culture was grown at 30 C for 18 to 24 h until clotted and used to inoculate a number of tubes of RSM which were stored at 4 C until required (<3 weeks). The last of the 4 C stock cultures was used to inoculate a further series of RSM tubes when required. No starter was subcultured more than 3 times before replacement with a new culture from the deep-frozen stock culture.

Non-Starter Lactic Acid Bacteria (NSLAB). Cultures of a pediococcus and a homolactic lactobacillus originally from cheese (see II.3 for characteristics and selection criteria) were obtained from Dr T.D.Thomas (New Zealand Dairy Research Institute). Stock cultures were stored frozen at -75 C partially grown in MRS broth (Difco Labs., Detroit, Mich., USA, after Man *et al*, 1960). At regular intervals, working cultures were obtained from the frozen stocks and maintained by subculture in RSM.

Sterile reconstituted skim milk (RSM). Sterile RSM for the growth of starter and NSLAB cultures was prepared as a 10% (w/v) solution of low-heat, antibiotic-free N.Z. skim milk powder in Milli-Q purified water (Millipore Corp., Bedford, Mass, USA). The required volumes were dispensed (either into flasks or 10 mL tubes) and sterilized by autoclaving at 115 C for 20 min.

II.2.2 Temperature Optimum determination. The optimum temperature for the growth of various NSLAB cultures was determined by growing the cultures in tubes in a temperature gradient incubator (Model TN3, Toyo Kagaku Sangyo, Co. Ltd., Tokyo, Japan). The method was that of Martley (1983) except that the growth medium was RSM supplimented with 0.5% yeast extract (BBL Biologicals Ltd.), and growth was for 18 h.

II.2.3 Cheesemanufacturing Methods.

II.2.3.1 Milk Supply. Milk for aseptic cheesemaking was obtained as described in Section I.A.4.1.1. For cheesemaking in open vats, milk was collected in 45 L cream cans from the bulk vat of the same farm, being representative of the milk from the remainder of the herd. This milk was combined night plus morning's milk, cooled and held at 7 C.

II.2.3.2 Aseptic Cheesemaking. Aseptic cheeses were made from aseptically drawn milk in the aseptic vats described in Section I.A.1. The manufacturing methods were as described

except that when cheeses were to be waxed, the cheeses were pressed in "10 lb" cheese hoops with cloth bandages. These hoops were sterilized as described for the rectangular hoops (I.A.1.4), filled within the vat and pressed inside cheese bags as previously described. The waxing method is described below (II.2.3.4).

II.2.3.3 Open Vat Manufacturing Methods. Cheeses were made in the 350 L rectangular open vats to the same manufacturing protocol of starter inoculum and rennet volumes, times and temperatures as in the aseptic vats, but the number of dry-stirs and the salting percentage were varied to keep the composition of the cheeses at 1 day as close as possible to those of the aseptic cheeses. The bulk starter was made in RSM sterilized by heating to >90 C for >1 h and cooled to 24 C. Culture was added and the bulk-starter cans incubated for 18 h at 24 to 22 C.

Raw milk was pasteurized at 72 C for 15 s using a PB13RB plate pasteurizer (Alfa Laval, Copenhagen, Sweden). To standardize the fat content of the vat milk, the vats were filled with the calculated proportions of whole and skim milk.

Following starter and rennet addition and coagulation, the coagulum was cut horizontally and vertically with 9 mm hand-held knives. From then until running, the curd was agitated using double-paddle agitators revolving at 14 rpm from an overhead drive. After running of the whey, the curd was dry-stirred and cheddared in the vats. Following milling

(through a reciprocating mill with a 9 mm cutter grid), salting and mellowing, the curd was hooped into rectangular 20 kg cheese hoops and pressed overnight at ambient temperatures. The 20 kg cheeses were cut into two equal blocks at 1 day and bagged and matured as for aseptic cheeses. For experiments with cheeses rinded and waxed, the salted cheeses were pressed in cylindrical "10 lb" cheese hoops overnight and then waxed.

II.2.3.4 Waxing of Cheeses. For cheeses which were waxed, two procedures were used. Cheeses which were to be waxed and bagged were cooled for 8 h at 10 C inside a cheese bag, the bag removed and the cheeses waxed by immersion in a heated bath of microcrystalline cheese wax (KW220, Jonk B.V., Koog Aan der Zaan, Holland) at 140 C. The waxed cheeses were placed in Cryovac shrink Barrier bags (B120, W.R.Grace Ltd., Porirua, New Zealand), the bags sealed under vacuum and shrunk by careful heating with hot water, ensuring that the wax itself was not melted. Following drying of the bag, the shrink bagged cheeses were further bagged and sealed under vacuum in a regular Writghcels bag. After three months storage, the shrink bag was removed and the cheeses rewaxed and rebagged but only in a Wrightcels bag.

For waxed and rinded cheeses (not bagged), each 4 kg cheese was held for 48 h at 12 C in a sterilized closed stainless steel bucket containing 500 g of self indicating silica gel to dry the rind (after Mabbitt et al, 1959). The operator wore

sterile gloves when handling the dehooped cheeses. Following the drying period, the cheeses were waxed as above and returned to the 12 C room with the waxed and bagged cheeses. The cheeses were rewaxed after 3 months of storage.

At the 14 day sampling time, the cheeses were moved to either the 10 or the 15 C maturing room for the remainder of the maturing period.

II.2.4 Slurry Manufacture. To rapidly check that aseptically manufactured cheeses did not contain NSLAB, 1 day old curd was made into cheese slurries as described by Kristoffersen et al (1967). Initially, slurries were incubated in sterilized polycarbonate bottles (Bel Art Products, Pequannock, NJ, USA.), however, these were replaced by cheese bags which were vacuum sealed and incubated in an air incubator at 30 C.

II.2.5 Sampling of the Cheeses. The cheeses were sampled using sterile microbiological techniques. The trier was dipped in alcohol, flamed and inserted into the cheese. The top 10-15 mm of the plugs used for plate counting were removed using an alcohol flamed spatula and the remainder of the plug placed in sterile petri dishes. Where samples were not required to be aseptic, they were still taken aseptically so as not to contaminate the cheese, and then placed into clean sample containers. Following sampling, each cheese was transferred to new bags and vacuum sealed.

For cheeses which had been waxed, the wax (and shrink bag) was sterilized by wiping with alcohol and an alcohol flamed scalpel was used to cut an opening in the wax so that the trier could be inserted. Samples were handled as above, but the plugholes were filled with melted cheesewax. The bagged cheeses were then rebagged in new outer bags and vacuum sealed.

II.2.6 Colony Counts. Cheese samples for microbiological analysis were weighed (10 g) into sterile wide-mouthed blending bottles and 90 mL of sterile 2% citrate added (final pH approx. 6.0). The samples were blended using an Ultraturrax (Janke and Kunkel GmbH, Staufen, West Germany) with 10N shafts. The shafts were sterilized by autoclaving (121 C/15 min) wrapped in aluminium foil.

Decimal dilutions were made in half-strength Ringers solution and 1 mL samples (in triplicate) were pour plated using the required agars.

Starter numbers were determined by plating in M17 agar (Terzaghi and Sandine, 1975) with aerobic incubation at 30 C for 36-48 h. NSLAB numbers were determined by plating in LBS agar (Rogosa SL agar, Difco Labs) with anaerobic incubation for 5 days at 30 C.

Coliform bacteria were enumerated in Violet Red Bile Agar (VRBA, Gibco Diagnostics, Madison, Wis., USA) incubated aerobically at 30 C for 24 h. The total count in raw and pasteurized milks was determined using Standard Methods Agar

(SMA, Gibco Diagnostics) incubated for 5 days aerobically at 30 C. Bacteria of the family Micrococcaceae were determined by plating in Baird Parker agar (Gibco Diagnostics, after Baird Parker, 1962) which contained Egg-yolk Tellurite solution (SR 64, Oxoid Ltd., London, UK). The plates were counted after 2 days aerobic incubation at 37 C.

All the agars were made and sterilized according to the manufacturers recommendations, and were dispensed in 100 mL quantities into 180 mL bottles (Kimax, Kimble Products, Toledo, Ohio, USA) before autoclaving. The sterile media were held at ambient temperatures in the sealed bottles until use. Before plating, sufficient bottles of agar were melted by heating at 121 C for 5 min and then tempered at 45 C. Additions of egg-yolk tellurite to Baird Parker agar were made to the tempered agars immediately before plate pouring.

Anaerobic atmospheres for agars grown anaerobically, were obtained by sealing the plates in cans and replacing the atmosphere by evacuation and refilling with an atmosphere of 5% CO_2 in hydrogen (New Zealand Industrial Gases, Wellington, NZ). The cans also contained an oxygen scavanging catalyst (Gaspak, BBL Microbiology Systems, Cockeysville, MD, USA).

II.2.7 Chemical Analyses.

The cheese were analysed for composition at one day old. Three plugs were drawn aseptically from each cheese and grated together before commencing the analyses.

Moisture. The moisture content of cheeses and the total solids content of milks were determined routinely using an Apollo Mark XII microwave moisture meter (Photovolt Corp., NY, NY, USA). Samples of approx 5 g where placed between two layers of glass-fibre filter paper, and heated for 3.5 min at power setting 15. Confirmatory tests were performed by the standard oven-heating method at 102 C for 18 h.

Fat. The fat content of milks was determined using a Milko-Tester Minor fat tester (A/S. N. Foss Electric, Hillerod, Denmark). Confirmatory tests were performed using the Babcock method (NZ Ministry of Agriculture Dairy Division Manual DDM 4.1.3a, 1979). Cheese fats were determined by the Analytical Chemistry Section, NZDRI, using the Babcock method.

pH. These were determined using a PHM82 pH meter (Radiometer Copenhagen, Denmark) with N61 combination pH electrodes (Schott Gerate, Hoffheim a Ts., GDR). For cheese, the grated sample was forced into a 5 mL glass beaker and the electrode inserted. No water was added.

The calibration of the electrode was checked frequently against standard buffers (pH 4.00 and 7.00) and only small batches of samples were analysed in one session.

Salt. Initially, salt in cheese was determined using the standard Volhard method (NZ MAF Dairy Division Manual, DDM 4.7.2a, 1979). However, during the course of the project, a "Memotitrator" (Mettler DL40RC, Mettler Instrumente AG, Greifensee, Switzerland) became available. Since this was shown to give identical results to the Volhard method with

much less effort, it became the method of choice. Approximately 2 g of cheese was accurately measured into the titrator cup, 100 mL of 1 M nitric acid added, and then heated for 30 min in a 60 C waterbath. After cooling to room temperature the chloride content was determined by equilibrium titration with standardized 0.12 M silver nitrate.

Calcium. The calcium content of the cheese was determined using the complexometric method (K.N. Pearce, 1977).

II.2.8 Texture and Flavour Assessment. Samples were taken for texture and flavour assessment after 3, 6 and 9 months of storage. For rectangular cheeses a 20-25 mm slab was cut from one end with a sterile wire and discarded. A 10 mm slab was then cut from the clean edge and 20 mm was removed from the edges. The sample was then bagged in a self seal plastic bag ("Whirlpak", Nasco, Fort Atkinson, Wis.,USA) and placed in a 12 C conditioning cabinet for 2 h before the tasting session. Cubes approximately 10 mm square were cut and presented to the tasters in individual ramdomly numbered cups. For the cylindrical, waxed cheese, plugs were drawn aseptically with a trier and 20 mm removed from the rinded surface. The samples were conditioned for 2 h at 12 C in Whirlpac bags before tasting. The plugs were cut into lengths of approx 20 mm for each taster. The details of the tasting proceedures are outlined in Section I.B.

II.3 Trials with Cheeses Matured in Bags.

II.3.1 Comparison of Cheeses Made in Aseptic Vats with Cheeses Made in Open Vats.

II.3.1.1 Statistical Approach. The effect of adventitious NSLAB on the texture and flavour of Cheddar cheese was investigated by manufacturing cheese in the aseptic vats from aseptically drawn milk and in open vats using milk from the same farm but not drawn aseptically. This experiment was repeated on 9 occasions over three seasons. Since only one cheese was made from each aseptic vat, two vats were used each day with the cheese from one vat being matured at 10 C and from the other at 15 C. The open vats required 300 L of milk and produced around 30 kg of cheese so for each experiment one 20 kg cheese was cut in half with one half matured at 10 C and the other at 15 C. Thus the aseptic vat cheeses were not a true split experiment while the open vat cheeses were. The data was therefore analysed by Analysis of Variance (Anova) in two ways, being the extremes of the possible error. The first approach assumes a true split plot on temperature given by the equation;

Replicate,Treatment/Temperature/Time

while the second assumes a nonsplit experiment, given by the equation;

Replicate,Treatment,Temperature/Time

The F-tests will only differ in the upper levels of the Anova and not affect time and the time interactions. As the non-split approach is likely to be the more conservative approach, the F-test data quoted in the text are from the non-split approach. Where the split plot approach gave F-test values which differed in significance, these are noted in the text. It should be noted that only the F-test values differ, not the actual means of the experimental data.

In the analysis of the taste panel results, the question arises of how to treat the subsamples due to the individual taster's results. If the subsamples are treated as error the number of degrees of freedom in the lowest plot of the Anova are increased by the total number of taster sessions involved (usually some hundreds). This increase causes very small differences in the time interactions to become highly statistically significant but, because of the split plot nature of the analysis, does not affect the treatment or temperature levels of the analysis.

Since these small differences were obviously not significant (and were shown not to be when subsamples were not treated as error), subsamples were treated separately from the error in all the analyses reported in this thesis.

II.3.1.2 Cheese Composition. The mean values for the composition of the aseptic vat and open vat cheeses are given in Table xii.

Table xii. Chemical composition of cheeses made in the aseptic and open vats.

	Aseptic		Open	
	Mean	s.d.[1]	Mean	s.d.
Moisture (%)	37.28	1.22	35.94	.64
Fat (%)	32.68	1.63	36.05	1.31
Calcium (mM/kg)	194	12	168	10
Salt (%)	1.60	.25	1.53	.20
pH	5.18	.11	5.08	.05
S/M (%)	4.66	.40	4.32	.54
MNFS (%)	54.39	2.04	55.70	1.28
FDM (%)	52.03	2.10	55.99	1.94
SNF (%)	30.04	.78	28.01	1.51
Ca/SNF (mM/kg)	628	39	602	48

[1] s.d. = Standard deviation.

The cheeses made in the aseptic vats were 1.6% higher in moisture than the cheeses made in the open vats while the fat content was 3.4% lower due to the greater fat loss from the aseptic vats (Section I.A.1.10). The MNFS and FDM of the cheeses from the aseptic vats was within the normal bounds for N.Z. Cheddar cheese while for the open vat cheeses these factors were about 1% higher than the maximum.

While the salt and calcium values appear somewhat different, the ratio of salt to moisture and calcium to SNF are similar (S/M 4.66 and 4.32, and Ca/SNF 628 and 602 respectively). The pH of the 1 day cheese differed by 0.10 units, being 5.18 in the aseptic vat cheeses and 5.08 in the open vat cheeses.

Despite attempts to obtain identical compositions in the cheeses from the two different vats, this was not obtained. However the differences observed may not have significantly affected the rates of maturation since the higher moisture content in the aseptic vat cheeses actually translates to a lower MNFS.

<u>II.3.1.3 Bacterial Counts</u>. The counts of starter and nonstarter bacteria in both aseptic vat and open vat cheeses are shown in Fig 12. In the aseptic vat cheeses matured at 15 C, the starter count decreased regularly from day 1 to reach levels of $1*10^6$ cfu/g at 3 months, but thereafter the count only slowly decreased to reach $2*10^5$ cfu/g at 9 months. In the cheeses matured at 10 C, the count reached $2*10^6$ cfu/g at 3 months and then continued to fall at a slower rate to reach $4*10^4$ cfu/g at 9 months. In the cheeses from the open vats, the count fell more slowly than in the aseptic vat cheeses reaching levels of $1*10^6$ cfu/g at 9 months. After about 1 month these counts reflected the high NSLAB counts in the cheeses and not the starter bacteria.

No NSLAB were detected in aseptic vat cheeses held at 10 C until 1 month. The count then rose slowly to reach maximum levels of 10^5 cfu/g at 6 months. At 15 C the count rose rapidly in the first 1 to 2 months to levels of 10^4 cfu/g, and then more slowly to reach levels of $3*10^6$ cfu/g by 9 months.

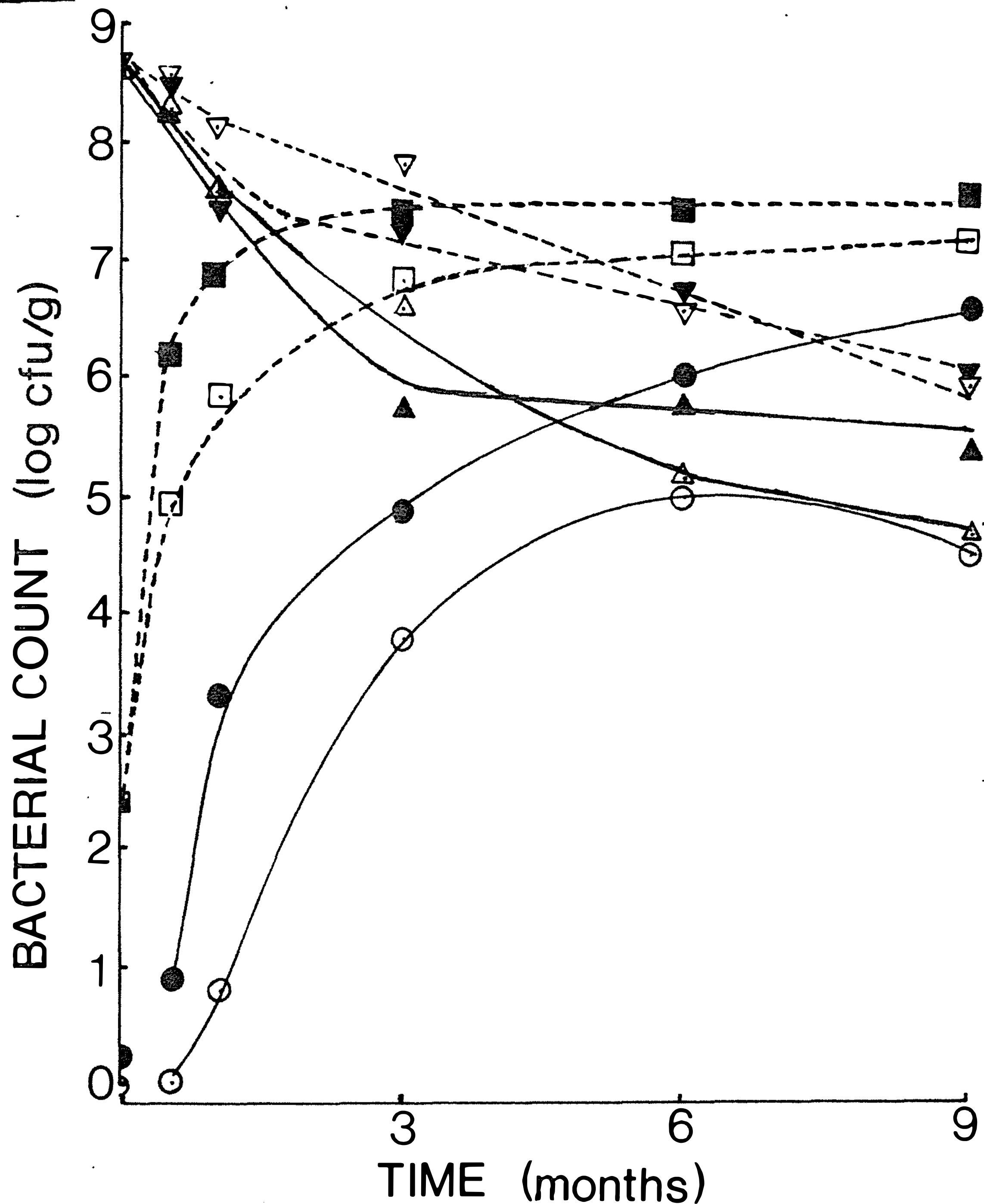


Figure 12. Counts of starter and NSLAB organisms in aseptically made cheeses and in open vat cheeses made from milk from the same farm. Starter and NSLAB counts were determined in cheeses made on the same day in either the aseptic vats from milk drawn aseptically, or in open vats from bulk milk from the same farm. The cheeses were matured at either 10 C or 15 C in barrier bags without waxing. △-▲, Starter in aseptic vat cheeses; ▽-▼, Starter in open vat cheeses; ⊙-●, NSLAB in aseptic vat cheeses; ⊡-■ NSLAB in open vat cheeses. Open symbols, 10 C; closed symbols, 15 C. Solid lines, aseptic vat cheeses; dashed lines open vat cheeses.

In the open vat cheeses the count at 1 day was 250 cfu/g. In the cheeses held at 15 C the count rose rapidly to reach levels of 10^7 cfu/g at 1 month and a plateau of $3*10^7$ cfu/g by 3 months. However, in the cheeses held at 10 C the count rose more slowly to reach $5*10^6$ cfu/g at 3 months and plateau at $1*10^7$ cfu/g by 6 months.

II.3.1.4 Texture and Flavour in the Cheeses held for 6 months at 10 and 15 C. The F-values and estimates of significance for the textural and flavour differences between the cheeses made in the aseptic vats and those made in the open vats are shown in Table xiii. The means of all the significantly different attributes are shown in Table xiv.

Texture. Aseptically made cheeses did not differ from open vat cheeses in any textural attribute except rubberiness (P<0.1%). Regardless of treatment, cheeses held at 15 C were more crumbly and sticky than cheeses held at 10 C (P<0.1 and P<5% respectively) and less rubbery (P<0.1%). At 6 months, firmness, rubberiness and mealiness were lower than at 3 months (P<0.1%, P<0.1% and P<5% respectively) while stickiness was higher (P<5%).

Two interactions were observed. For crumbliness, a treatment by temperature interaction indicated that the cheeses from the aseptic vats increased more between 3 and 6 months than did the cheeses from the open vats (Table xv),

while for stickiness, a temperature by time interaction indicated that the cheeses at 10 C increased in stickiness while the cheese at 15 C did not.

Table xiii. F-values and estimates of significance of texture and flavour attributes of cheeses made in the aseptic and open vats and held for 6 months at 10 C and 15 C.

	Rep[1]	trt	Temp	trt*T	/time	trt*t	T*time	trt*T*t
Firmness	[2]				***[3] 19.78			
Rubbery		*** 14.12	*** 36.32		*** 28.95			
Crumbly	* 2.90		*** 18.25	** 8.18				
Smooth					* 6.53			
Sticky			* 4.68				* 5.25	
Mealy					* 5.95			
Gritty								
Acid/sour			*** 32.22					
Fruity	* 2.66		*** 24.28		*** 27.58		** 10.92	
Sulphide		* 5.63	*** 76.83		*** 61.15		*** 51.22	* 4.69
Sharp		* 4.68	*** 45.56		*** 71.99		*** 22.70	
Bitter	* 2.51		*** 22.15					

[1] Rep = Replicate, trt = treatment, Temp = Temperature, t = time.
[2] For clarity, the data for non-significant factors have been omitted.
[3] * = P <5%, ** = P <1%, *** = P <0.1%

Table xiv. Significant means of the differences in texture and flavour attributes of cheeses made in the aseptic (AV) and open (OV) vats and held for 3 and 6 months at 10 C and 15 C.

	Treatment		Temperature		Time	
	AV	OV	10 C	15 C	3 mo	6 mo
Firmness			4.93	4.65	4.98	4.59
Rubbery	1.85	1.37	2.00	1.22	1.84	1.37
Crumbly			2.06	2.77		
Smooth					4.54	4.81
Sticky			4.05	4.47		
Mealy					1.93	1.66
Gritty						
Acid/sour			4.34	5.01		
Fruity			2.75	3.44	2.75	3.45
Sulphide	1.43	1.95	.68	2.70	.98	2.40
Sharp	1.56	1.94	1.16	2.34	1.27	2.25
Bitter			.49	.85		

When the analysis of variance was performed with a split on temperature (the other extreme of the statistical approach), a number of differences in the F-values of significance were noted (data not shown). The effect of replicate on crumbliness was reduced to insignificance, while that of treatment on rubberiness was reduced to significant at P<5%. For firmness, temperature became significant (P<1%) while for mealiness the temperature effect increased in significance to P<1%.

Three more treatment by temperature interactions became significant. The F-test indicated that aseptic vat cheeses did not change in firmness while the open vat cheeses had reduced firmness at 6 months (P<1%). For rubberiness (P<1%) there was a greater decrease in aseptic vat cheeses than in

open vat cheeses, while for smoothness ($P<5\%$), the aseptic vat cheeses decreased between 3 and 6 months while the open vat cheeses increased.

Flavour. Overall, both sulphide and sharpness were higher in open vat cheeses than in aseptic vat cheeses ($P<5\%$, Table xiv). At 15 C, all the five attributes were significantly higher than at 10 C ($P<0.1\%$), while at 6 months fruitiness, sulphide and sharpness were higher than at 3 months ($P<0.1\%$).

There were no significant treatment interactions, however there were significant temperature*time interactions for

Table xv. Interaction means of the differences in texture and flavour attributes of cheeses made in the aseptic (AV) and open (OV) vats and held for 3 and 6 months at 10 C and 15 C.

Treatment*Temperature

	Firmness		Rubberiness		Crumbliness		Smoothness	
	AV	OV	AV	OV	AV	OV	AV	OV
10 C	4.66	5.19	2.37	1.62	1.80	2.32	4.77	4.62
15 C	4.64	4.65	1.33	1.11	2.98	2.56	4.36	4.94

Temperature*Time

	Stickiness		Fruitiness		Sulphide		Sharpness	
	3 mo	6 mo	3 mo	6 mo	3 mo	6 mo	3 mo	6 mo
10 C	3.85	4.26	2.62	2.89	.60	.74	.95	1.38
15 C	4.47	4.46	2.89	4.00	1.33	4.04	1.58	3.11

fruitiness, sulphide and sharpness (Table xv) with P<1%, P<0.1% and P<0.1% respectively. In all three cases, the intensity increased more rapidly in cheese held at 15 C than in cheese held at 10 C.

While the only significant three-way interaction was for sulphide (P<5%), the three-way interactions for fruitiness, sharpness and bitterness show the same trends and are included in Table xvi. In all three cases, the open vat cheeses developed higher intensities than aseptic vat cheeses especially at 15 C.

Table xvi. Threeway interactions for the differences in texture and flavour attributes of cheeses made in the aseptic (AV) and open (OV) vats and held for 3 and 6 months at 10 C and 15 C.

	Fruity		Sulphide		Sharp		Bitterness	
	3 mo	6 mo	3 mo	6 mo	3 mo	6 mo	3 mo	6 mo
AV 10 C	2.50	2.92	.49	.67	.82	1.24	.40	.51
AV 15 C	2.78	3.92	1.23	3.22	1.53	2.68	.69	.86
OV 10 C	2.73	2.87	.71	.80	1.08	1.53	.59	.46
OV 15 C	3.00	4.08	1.44	4.86	1.64	3.54	.73	1.12

When the data was analysed with a split on temperature, the effect of replicate on both fruitiness and bitterness became insignificant as did the treatment effect on sharpness.

<u>II.3.1.5 Texture and Flavour of Cheeses Held for 9 months at 10 C</u>. Only the 10 C cheeses were analysed at 9 months, and therefore the data for cheese at 10 C was reanalysed seperately using the Anova:

Replicate,Treatment/Time

The results are presented in Tables xvii and xviii.

<u>Table xvii. F-values and estimates of significance of texture and flavour attributes of cheeses made in the aseptic (AV) and open (OV) vats and held for 9 months at 10 C</u>.

	Rep[1]	trt	time	trt*t
Firmness[2]				
Rubbery		* 5.33	*** 23.89	* 4.07
Crumbly			* 8.16	
Smooth				
Sticky			*** 20.07	
Mealy				
Gritty				
Acid/sour			*** 11.76	
Fruity			*** 15.84	
Sulphide			*** 23.32	
Sharp			*** 32.76	
Bitter			** 5.36	

[1] Rep = Replicate, trt = treatment, t = time
[2] For clarity, data for insignificant factors have been omitted
[3] * = P < 5%, ** = P < 1%, *** P < 0.1%

Again time was highly significant, especially for flavour development, however treatment (aseptic or open vat manufacture) did not affect flavour, with the only effect being a slightly lower level of rubberiness in the open vat cheeses (P<5%).

Table xviii. Significant means of the differences in texture and flavour attributes of cheeses made in the aseptic (AV) and open (OV) vats and held for 9 months at 10 C.

	Treatment		Time		
	AV	OV	3mo	6 mo	9 mo
Firmness					
Rubbery	2.08	1.54	2.28	1.71	1.43
Crumbly			2.11	2.01	2.71
Smooth					
Sticky			3.85	4.26	4.82
Mealy					
Gritty					
Acid/sour			4.25	4.43	4.95
Fruity			2.62	2.89	3.64
Sulphide			.60	.74	2.22
Sharp			.95	1.38	2.37
Bitter			.49	.49	.90

II.3.2 Source of NSLAB in Control Aseptic Vat Cheeses.

Despite extensive testing, no source of NSLAB infection was detected during cheese manufacture. Slurries made from 1 day aseptic vat cheeses suggested by interpolation, that the contamination level was about 1 cfu/kg of cheese (Huffman and Turner, unpublished results). However on 4 occasions,

slurries made from aseptic vat cheeses contained no NSLAB at 10 days, while the cheeses contained NSLAB after 35 days maturing.

The results from two of the cheeses which were control cheeses in the aseptic vat comparison trial with open vat manufacture (Section II.3.1) are shown in Table xix. While no NSLAB were detected in the slurries after either 5 or 10 days at 30 C, both cheeses at 35 days had low level NSLAB contamination. In the cheeses at 10 C a plateau of around 10^4 cfu/g was reached by 3 months, but in the cheeses at 15 C the count continued to rise to reach $2*10^7$ cfu/g by 9 months.

Table xix. NSLAB counts during the maturing of two cheeses made in the aseptic vats and in slurries made from their curds when 1 day old.

Cheese Number	Slurry 5 d[1]	Slurry 10 d	Cheese 1 d	14 d	35 d	3 mo	6 mo	9 mo
157	<1[2]	<1	<1	<1	24	$1*10^4$	$3*10^4$	300
158	<1	<1	<1	<1	$1*10^4$	$7*10^5$	$5*10^6$	$2*10^7$

[1]d = days, mo = months
[2]counts in cfu/g

Since the contamination of these cheeses must have occurred after pressing (at day 1) the outside of some mature control cheeses was carefully removed and sampled and a sample also taken from the inside of the cheese. The counts of 5 cheeses, split at 1 day and held at both 10 C and 15 C are shown in Table xx, along with counts from three open vat cheeses

obtained by sampling in the same manner. In the aseptic vat cheeses held at 10 C, there was no contamination of the inside of the cheeses while the outside of the cheeses had log mean levels of $8.2*10^7$ cfu/g. In the companion cheeses held at 15 C the levels were: inside $1.5*10^7$ cfu/g, outside $1.0*10^8$ cfu/g. In comparison, the open vat cheeses at 10 C

Table xx Interior and exterior NSLAB counts of 9 month cheeses.

Cheese No	10 C Inside	10 C Outside	10 C Plugged	15 C Inside	15 C Outside	15 C Plugged
Aseptic Vat Cheeses						
168	3	$2*10^8$	300	ND[1]	ND	$2*10^6$
174	<1	$5*10^7$	$3*10^4$	ND	ND	$3*10^6$
175	<1	$6*10^7$	$3*10^7$	$1*10^7$	$1*10^8$	$4*10^6$
178	1	$7*10^7$	$2*10^4$	$3*10^7$	$8*10^7$	$6*10^6$
180	<1	$1*10^8$	$3*10^4$	$1*10^7$	$1*10^8$	$3*10^7$
Mean	NA[2]	$8*10^7$	$5*10^4$	$2*10^7$	$1*10^8$	$6*10^6$
Open Vat Cheeses						
OV 7	$1*10^6$	$3*10^8$	$2*10^7$	$4*10^6$	$3*10^8$	$2*10^7$
OV 8	$3*10^5$	$7*10^7$	$7*10^6$	$9*10^6$	$7*10^7$	$3*10^7$
OV 9	$8*10^4$	$3*10^7$	$6*10^6$	$3*10^6$	$1*10^7$	$2*10^7$
Mean	$3*10^5$	$9*10^7$	$8*10^6$	$5*10^6$	$6*10^7$	$2*10^7$

[1] ND = not determined.
[2] NA = not appropriate since logl = 0.

differed by over 2 logs while at 15 C the difference was 1 log. Apparently, the cheese surfaces had become contaminated with NSLAB and the interface between the bag and the cheese enabled the contamination to spread around the cheese. One method to reduce this surface contamination would be to wax the cheese surface before bagging.

II.3.3 Conclusions.

II.3.3.1 Physical and Bacterial Composition. The cheeses made in the aseptic vats were higher in moisture but lower in fat than the corresponding open vat cheeses. As a result, they were actually lower in both MNFS and FDM. The lower MNFS would be expected to reduce the rate of maturation in the aseptic vat cheeses. It is unlikely that any flavour differences between the aseptic vat and open vat cheeses could be due to the differences in composition.

II.3.3.2 Texture and Flavour. The texture of aseptic vat and open vat cheeses was influenced by both the storage temperature and the maturing time. With time, cheeses became less rubbery but crumblier. At 15 C cheeses were less rubbery than cheeses at 10 C. Firmness was never affected by either temperature or time, while smoothness sometimes increased and sometimes decreased. Stickiness and grittiness occasionally differed but never consistently. Mealiness increased with time in the 6 month trials but in the cheeses held at 10 C for 9 months, no effect was seen. Similarly, mealiness decreased

in the deliberately added NSLAB trial, but there was no difference in the trial comparing aseptic and open vat cheeses.

In the trial comparing aseptic with open vat cheeses, the aseptically made cheeses were consistently more rubbery than the open vat cheeses. This was probably due to the higher SNF in the aseptic cheeses and was not found in the cheeses with deliberate NSLAB addition.

All 5 flavour attributes increased with temperature and most were also increased by time. In many cases, the temperature by time interaction was also significant, showing that the effect of time was different (greater) at 15 C than at 10 C. In open vat cheeses, the sharpness and sulphide characteristics were higher than in the aseptic vat cheeses, indicating that the NSLAB increased the intensities of these flavour attributes.

The trend in the threeway interactions indicated that the increase in flavour intensities was almost entirely in the cheeses held at 15 C, a fact confirmed by the analysis of the cheeses held to 9 months at 10 C where no treatment effects were observed.

These data indicate that the effect of adventitious NSLAB was only detectable when the cheeses were held at a very high temperature (15 C) for more than 3 months.

<u>II.3.4.3 Surface Contamination</u>. Despite the pressing of the aseptically made cheeses inside cheesebags, the sampling of the cheeses in the more enclosed environment of the laboratory, and the use of bags which apparently were not contaminated with NSLAB (see Section I.A.4.3), all the cheeses matured in bags developed a contaminating flora of adventitious NSLAB on the surface. Apparently, the cheeses held at 15 C developed an internal flora, however this was most probably due to the entry of the surface organisms down the plugholes from previous sampling times and through the fissures which opened in the cheeses due to their extended incubation at such a high temperature.

The presence of higher counts of NSLAB on the surface of even open vat cheeses indicates that all cheese surfaces (including internal fissures) are likely to have increased NSLAB counts. This is probably due to the fact that the colonies are not bound in the cheese matrix but are free to spread across the entire surface. Where conditions are suitable, these bacteria may be involved in undesirable reactions like bleaching and intense sulphide production (Barnicoat, 1950). Potentially, if the barrier bag around the cheese is not sufficiently impermeable, the surface flora could be involved in aerobic metabolism like that proposed by Thomas (1987a), causing surface flavour defects.

While the presence of the contaminating flora lowered the validity of the results, and therefore required a further trial with cheeses whose surface was protected by a thin

coating of wax (Section II.4), it must still be noted that the differences reported in this section were all found with cheeses with this surface flora, and so occurred despite that flora.

II.4. Trials with Waxed Cheeses.

II.4.1 Statistical Approach. Since the aseptic vats produced only two 4 kg cheeses, it was necessary to continue the incomplete split-plot analytical approach. Each aseptic vat was used to produce two cheeses, one was waxed and bagged, the other waxed and matured rinded. Both cheeses from one vat were matured at the same temperature. The open vat was used to produce four cheeses so that a true split was possible with two cheeses waxed and bagged and two waxed and rinded. One of each was then matured at each temperature. This experiment was conducted four times in February and March 1986.

The data was analysed in two ways, being the extremes of the possible error inherent in the partial split. To analyse as a true split on temperature experiment would require the Anova to be:

Replicate,Treatment /Surface treatment /Temperature /Time

To analyse with no split on temperature the Anova would be:

Replicate,Treatment,Temperature /Surface treatment /Time

The F-tests will only differ in the upper plots since each split is tested against the error in that split. Where the F-test values differed between the two approaches they are noted in the text.

II.4.2 Cheese Composition. The composition of the cheeses is shown in Table xxi. The moisture, salt and salt-in-moisture of aseptic vat and open vat cheeses were identical. However

the open vat cheeses were 1.1% higher in fat and therefore lower in SNF (by 1.2%). This resulted in a 1-2% increase in both the MNFS and FDM in the open vat cheeses, however both were within normally accepted limits for Cheedar cheese. The calcium level in open vat cheeses was slightly lower (by about 3%) however on a calcium/SNF basis, the calcium levels were identical.

Table xxi. Chemical composition of cheeses made in the aseptic and open vats during the comparison with waxed cheeses.

	Aseptic		Open	
	Mean	s.d.[1]	Mean	s.d.
Moisture (%)	36.20	1.04	36.12	1.26
Fat (%)	32.8	1.78	33.9	1.54
Salt (%)	1.71	0.16	1.70	0.18
Calcium (mM/kg)	5	6	179	12
MNFS (%)	53.48	0.83	54.62	1.29
FDM (%)	51.12	2.07	53.02	1.04
S/M (%)	4.72	0.48	4.73	0.59
SNF (%)	31.28	1.00	30.01	1.05
Ca/SNF (mM/kg)	600	34	597	26
pH	5.09	0.06	5.05	0.05

[1] s.d. = standard deviation.

The open vat cheeses had a slightly lower pH at 1 day, however as all the cheeses matured the pH rose (Table xxii). The pH of the open vat cheeses held bagged at 15 C rose more rapidly than the aseptic vat cheeses at 15 C, while the cheeses held at 10 C (either aseptic or open vat) rose only to 5.15 at 6 months.

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Table xxii. The change in pH of aseptic (AV) and open (OV) vat cheeses waxed and matured either bagged (BAG) or rinded (WAX) at 10 C and 15 C.

			1 Day		3 months		6 months	
			Mean	s.d.[1]	Mean	s.d.	Mean	s.d.
AV	10 C	WAX	5.09	0.08	5.15	0.01	5.17	0.03
		BAG	5.09	0.08	5.15	0.04	5.15	0.03
	15 C	WAX	5.08	0.04	5.15	0.04	5.17	0.01
		BAG	5.08	0.04	5.15	0.02	5.28	0.08
OV	10 C	WAX	5.05	0.05	5.08	0.05	5.12	0.05
		BAG	5.05	0.05	5.10	0.06	5.15	0.07
	15 C	WAX	5.05	0.05	5.13	0.06	5.37	0.13
		BAG	5.05	0.05	5.18	0.05	5.43	0.26

[1] s.d. = standard deviation.

II.4.3 Bacterial Counts. The counts of starter in aseptic vat cheeses and NSLAB in open vat cheeses are shown in Fig 13. The starter counts in open vat cheeses were not measured as after 1 month the NSLAB have grown to such counts in the cheeses that they outnumbered the starter on M17 agar. NSLAB organisms were absent (<1 cfu/g) except that surface contamination was found at the 6 month sampling time on two of the four aseptically manufactured cheeses waxed and bagged and matured at 15 C.

The starter count in aseptic vat cheeses decreased rapidly in the cheeses held at 15 C reaching levels of 10^5 cfu/g at 3 months and 10^4 cfu/g at 6 months. At 10 C however the decrease was much slower, with levels of 10^7 cfu/g being found at 3 months and $2.5*10^5$ cfu/g at 6 months.

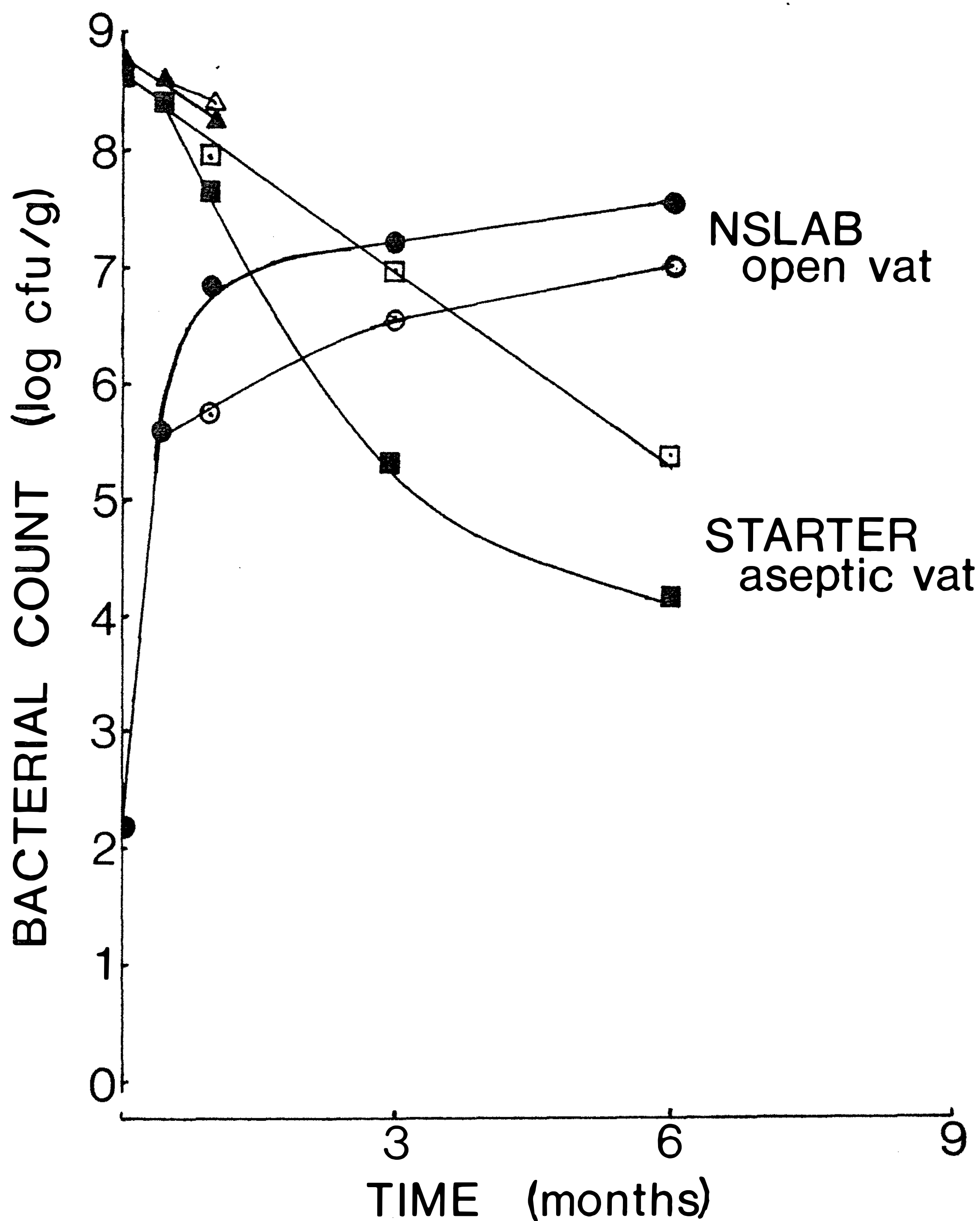


Figure 13. Starter and NSLAB counts in aseptic and open vat cheeses matured either rinded and waxed or waxed and bagged. Starter and NSLAB counts were determined in cheeses made on the same day in either the aseptic vats from milk drawn aseptically, or in open vats from bulk milk from the same farm. The cheeses were matured at either 10 C or 15 C in either barrier bags with waxing of the cheese surface before bagging, or rinded and waxed. ⊡-■, Starter in aseptic vat cheeses; △-▲, starter in open vat cheeses; ⊙-● NSLAB in open vat cheeses (no NSLAB were found in aseptic vat cheeses). Open symbols, 10 C; closed symbols, 15 C.

The NSLAB count in the open vat cheeses rose rapidly from 150 cfu/g at 1 day to $3.5*10^5$ cfu/g in the first 14 days (when the cheeses were at 12 C). After that time, the NSLAB count in the cheeses at 15 C continued to rise rapidly reaching levels of $2*10^7$ cfu/g by 3 months. In the cheeses held at 10 C the count had only reached 10^7 cfu/g at 6 months.

II.4.4 Texture and Flavour. The F-values and estimates of significance for the textural and flavour differences between the cheeses made in aseptic or in open vats, matured either bagged or rinded, stored at 10 C and 15 C and measured after 3 and 6 months are shown in Table xxiii. The means of all the significant factors are shown in Table xxiv.

II.4.4.1 Texture. Aseptic vat cheeses were less crumbly than open vat cheeses but also smoother and stickier (all P<5%). Rinded cheeses were less smooth (P<5%) and more gritty (P<5%) than bagged cheeses. When the Anova was performed without splitting, firmness also showed as being higher in rinded cheeses (P<5%).

Rubberiness decreased with storage temperature (P<5%), crumbliness increased with temperature (P<5%) and time (P<0.1%), and stickiness with time (P<1%) regardless of treatments. There were no significant interactions between the various factors.

Table xxiii. F-values and estimates of significance of texture and flavour attributes of cheeses made in the aseptic and open vats and matured either waxed or bagged held for 6 months at either 10 or 15 C.

	Rep[1]	z/bw	zb/T	zT	bT	zbT/t	zt	bt	zbt	Tt	zTt	bTt	zbTt
Firmness [2]													
Rubbery				*** [3] 32.30									
Crumbly	* 12.98			* 8.48			*** 65.31						
Smooth	* 14.51	* 7.43											
Sticky	* 17.54						** 8.71						
Mealy													
Gritty		** 16.70											
Acid/sour				*** 49.54			** 12.30						
Fruity	* 17.77			*** 96.09			*** 29.33					*** 22.37	
Sulphide				*** 32.43			*** 44.98					** 13.06	
Sharp				*** 77.14	* 5.15		*** 113.64	* 4.92				** 9.53	
Bitter	* 27.54			* 7.00			*** 38.39						

[1] Rep = Replicate, z = treatment, bw = Bagged or waxed (rinded), T = Temperature, t = time.
[2] For clarity, the data for non-significant factors has been omitted.
[3] * = P < 5%, ** = P < 1%, *** = P <0.1%

II.4.4.2. Flavour. All the attributes were significantly increased by both temperature and time. The significance was P<0.1% in all except two cases - bitterness with temperature (P<5%) and acid with time (P<1%). Treatment (aseptic vat versus open vat manufacture) only affected fruitiness, with the aseptic vat cheeses being less fruity than open vat

cheeses (P<5%). Surface treatment did not affect any of the flavour attributes. There was a slight replicate effect on bitterness (P<5%), with one replicate scoring consistantly higher for bitterness than the others. The intensity levels were less than 2.0 (Ui.e.u below threshold).

Table xxiv. Means of the significant differences in texture and flavour attributes of cheeses made in the aseptic (AV) and open (OV) vats and matured either waxed and bagged (BAG) or waxed and rinded (WAX) and held for 3 and 6 months at either 10 or 15 C.

	Treatment		Storage		Temperature		Time	
	AV	OV	BAG	WAX	10 C	15 C	3 mo	6 mo
Firmness [1]								
Rubbery					1.82	1.31		
Crumbly	2.10	2.71			2.17	2.63	1.92	2.92
Smooth	4.95	4.38	4.82	4.53				
Sticky	4.51	4.10					4.20	4.42
Mealy								
Gritty			.02	.06				
Acid/sour					4.35	4.99	4.53	4.81
Fruity	2.72	3.06			2.54	3.25	2.69	3.10
Sulphide					.66	1.77	.67	1.80
Sharp					.99	1.80	.94	1.87
Bitter					.49	.73	.31	.94

[1] For clarity, the data for non-significant factors has been omitted.

A number of attributes interacted at significant levels. Treatment (AV or OV) interacted with both temperature and time for sharpness (both P<5%) with the open vat cheeses showing greater intensities at the higher temperature or longer time (Table xxv).

There was also a temperature*time interaction for the fruitiness, sulphide and sharpness attributes (Table xxvi). The cheeses held at 15 C showed a more rapid increase in intensity than did the cheeses held at 10 C.

Table xxv. The differential effect of type of manufacture (aseptic or open vat) with either temperature or time of maturation on the sharpness of cheese.

	Trt[1]*Temp		Trt*Time	
	10 C	15 C	3 months	6 months
Aseptic vat	.93	1.54	0.86	1.63
Open vat	1.04	2.09	1.02	2.13

[1] Trt = Treatment

While neither the 3-way nor the 4-way interactions were significant, trends can be discerned from the plotted data for the fruity, sulphide and sharp attributes (Fig 14). There was little difference between the cheeses held at 10 C whether aseptic or open vat, bagged or rinded, and all increased slightly with time. However at 15 C, intensities in the bagged cheeses increased more with time than in the waxed cheeses, and intensities in the open vat cheeses increased more than in the aseptic vat cheeses. In all cases, the open vat bagged cheeses had the highest intensities of all treatments at 6 months of age.

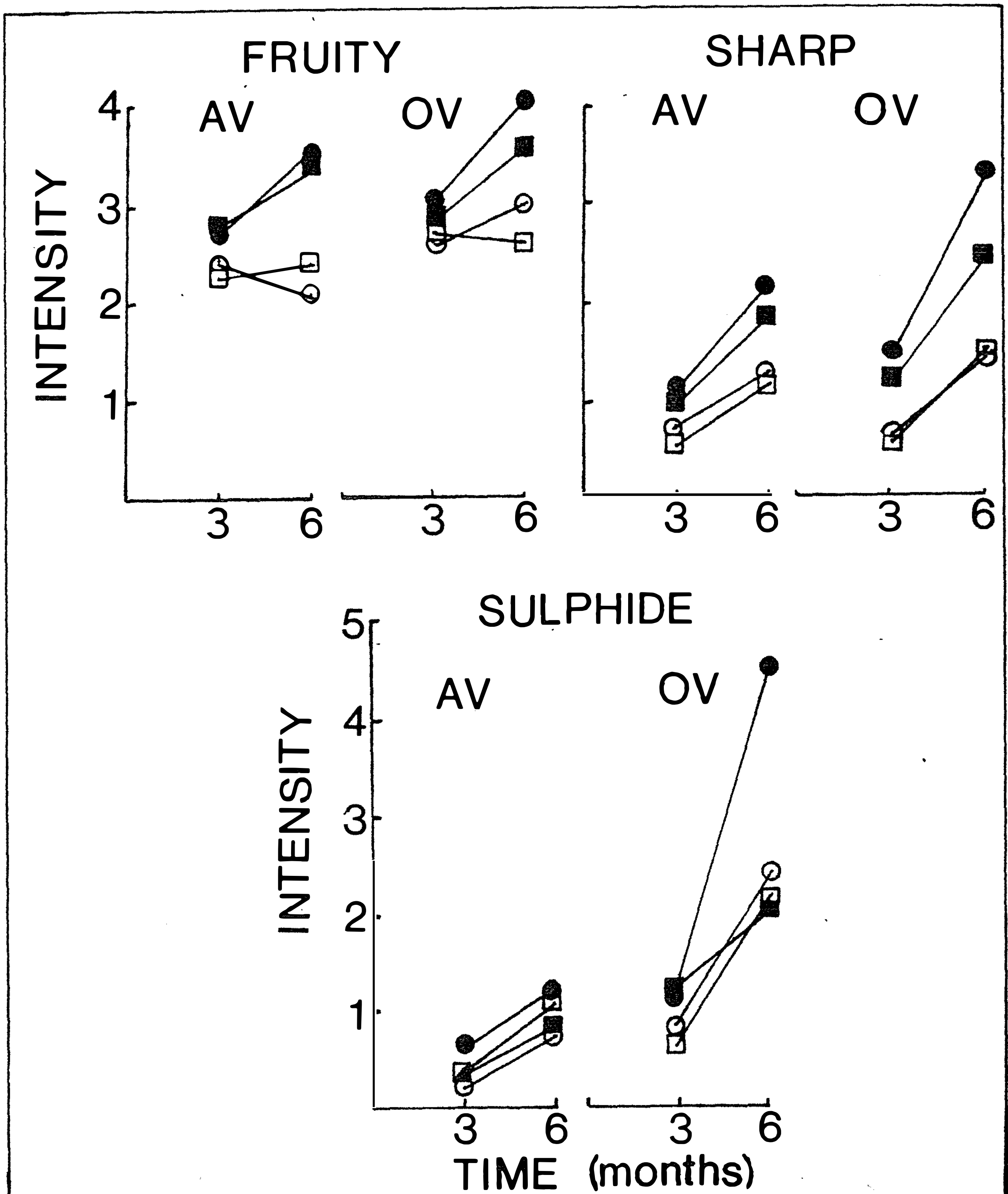


Figure 14. Threeway interaction means for fruity, sharp and sulphide attributes of the flavours of aseptic and open vat cheeses matured either rinded and waxed or waxed and bagged. The threeway interaction means of the flavour attributes of aseptic and open vat cheeses matured either waxed and bagged or waxed and rinded were determined by analysis of variance. The mean values of four replicates, for the attributes fruity, sharp and sulphide are shown at 3 and 6 months. ʘ - ●, waxed and bagged; ⊡ - ■, rinded and waxed. Open symbols, 10 C; closed symbols, 15 C.

Table xxvi. Temperature*time interaction means for fruitiness, sulphide and sharp of cheeses made in the aseptic and open vats and matured either waxed or bagged held for 3 and 6 months at either 10 or 15 C.

	10 C	15 C
	Fruitiness	
3 mo	2.51	2.87
6 mo	2.57	3.67
	Sharp	
3 mo	.65	1.23
6 mo	1.34	2.43
	Sulphide	
3 mo	.40	.94
6 mo	.95	2.70

II.4.5 Conculsions. The waxing of the cheeses before bagging reduced the surface contamination virtually to zero. Therefore, any conclusions drawn are not clouded by the presence of contaminating surface NSLAB contamination as was a problem with the previous section.

The moisture, salt and calcium contents and the pH at 1 day of the cheeses from the two treatments (aseptic or open vat manufacture) were almost identical. The open vat cheeses had a 1% higher fat content and this was reflected in a 1% higher MNFS, and a 2% higher FDM. Interestingly, the panel assessment of the texture was that the aseptic vat cheeses were less crumbly, smoother and stickier, when from the composition, one might expect the reverse.

The open vat cheeses consistently had higher fruitiness levels (P<5%) than the aseptic vat cheeses. This attribute showed significance due to the narrow range of scores given by the panel. Other attributes differed by similar amounts but were not found to be significant due to the wider spread of intensity values given by the panel.

The level of sharpness increased more rapidly in open vat cheeses than in aseptic vat cheeses with both temperature and time. The threeway interactions (though not statistically significant) showed that this effect was almost entirely due to maturing at 15 C, since the intensities in the cheeses held at 10 C were identical, as were the intensities in the cheeses at 15 C after 3 months.

Due to the wide range of scores ascribed to the intensity of sulphide, this attribute rarely attained significance statistically. However, the data for the threeway interactions indicated that there was a difference at 6 months between open vat and aseptic vat cheeses, with the open vat cheeses having higher sulphide levels. This may have contributed to the detection of higher sharpness levels since sharpness is associated with the mature flavour of which sulphide is a part. The threeway interactions also showed that the bagged cheeses had higher levels of sulphide than the waxed cheeses. Whether this was due to greater production of sulphurous compounds in the bagged cheeses, or to the greater retention of these compounds by the presence of the bag is unknown.

The results of this section confirm the results of Section II.3, but with the added certainty due to the absence of the surface contamination found in the unwaxed cheeses. The presence of NSLAB did not affect the texture or flavour of cheeses at either 10 C or 15 C in 3 months, but when the cheeses were held for 6 months at 15 C, the NSLAB, whether adventitiously or deliberately added, increased the sharpness and sulphide characteristics.

II.5. The Effect of NSLAB Addition to Aseptically Made Cheeses.

II.5.1 Introduction. Previous attempts to introduce cultured NSLAB into cheese produced atypical flavours (Sherwood, 1939c; Reiter et al, 1967). On the assumption that these flavours were produced because of the method of culturing and inoculation of the NSLAB, methods were devised to introduce NSLAB cultures in a way that would mimic the growth of post-pasteurization adventitious contamination.

Representative cultures of the NSLAB isolates held at the NZDRI were selected (Section II.5.2), cultured in reconstituted skim milk (Section II.5.3) and inoculated into aseptically manufactured cheeses (Section II.5.4).

Having established that the organisms could be sucessfully introduced and grew at rates similar to those found naturally, a trial was conducted to determine the effect of two representative strains on the texture and flavour formation in the cheeses (Section II.5.5). Unfortunately, this trial was completed before the surface contamination problem was resolved, and the results are confounded by the growth of adventitious NSLAB on the cheese surface. Since little effect of NSLAB was found in this trial, or in the trials reported in Sections II.3 and II.4, these trials were not repeated without surface contamination.

II.5.2 Characteristics of NSLAB cultures. To select representative NSLAB, the culture collection of the NZDRI was checked to determine what types of lactobacilli and pediococci had been isolated from cheese. Forty isolates were examined, comprising 15 isolated from N.Z. Cheddar cheeses and deposited before 1973 by G. Hogland, 3 from NZ Cheddar cheeses and 13 from large block American Cheddar cheeses isolated by Dr. T.F.Fryer about 1976, and 9 recent isolates from Dr. T.D.Thomas also from NZ Cheddar cheeses but from a different manufacturing plant.

Of the 40 isolates tested, 23 were homolactic lactobacilli, 10 heterolactic lactobacilli and 7 Pediococci.

II.5.2.1 Homolactic lactobacilli. The characteristics of the homolactic lactobacilli are shown in Table xxvii. As all grew at 15 C they were not of the thermophilic group of strains. In MRS broth, 5 produced only L-lactic acid (indicative of *L. casei*), 3 produced only D-lactic acid and 15 produced DL-lactic acid (indicative of *L. plantarum*, Rogosa, 1974).

Three isolates were selected for cheesemaking trials on the basis of their differing production of D-lactate. Isolate 43 produced DL-lactate, isolate 44 produced D-lactate and isolate 45 produced L-lactate. All were able to metabolize lactose and clot either milk or yeast-milk and none utilized citrate. Isolate 38 was rejected (although some information has been published (Thomas and Crow, 1983) as it appeared to ferment citrate.

II.5.2.2 Heterofermentative lactobacilli. The characteristics of the heterolactic lactobacillus cultures are shown in Table xxviii. All were isolated from Cheddar cheeses made in NZ, one made in the NZDRI and 9 commercially manufactured.

Table xxvii. Growth characteristics of various homolactic lactobacillus cultures originally isolated from Cheddar cheeses.

Culture	Gas in MRS[1]		Growth in MRS at		Growth in[2]			%
	Std	2% cit	15C	45C	M17	RSM	YM	D lac[3]
1	-[4]	-	+	-	+	-	+	73
7	-	-	+	-	+	-	+	77
8	-	-	+	-	+	-	+	85
9	-	-	+	-	+	-	+	81
10	-	-	+	-	+	-	+sl	58
11	-	-	+	-	+	-	+	70
12	-	-	+	-	+	-	+	45
13	-	-	+	-	+	-	+	68
23	-	-	+	-	+	+	nd	39
28	-	-	+	-	+	+	nd	17
29	-	-	+	-	+	+sl	+	71
31	-	-	+	-	+	-	+	78
32	-	-	+	-	+	+sl	+	0
33	-	-	+	-	+	-	+	93
34	-	-	+	-	+	+	nd	100
35	-	-	+	-	-	-	-	?
36	-	-	+	-	+	+sl	nd	0
38 L4	-	+	+	-	+	-	+	62
42	-	-	+	-	-	-	+sl	60
43 L14	-	-	+	-	+	-	+	83
44	-	-	+	-	+	-	+	91
45	-	-	+	-	+	+	nd	0
46	-	-	+	-	-	-	-	0

[1] MRS = Man, Rogosa and Sharpe liquid medium, Std = with normal citrate concentration, 2% cit = with 2% citrate.
[2] Liquid media, M17 = medium of Terzaghi and Sandine (1975) with lactose as energy source, RSM = reconstituted skim milk, YM = RSM with 0.5% added yeast extract
[3] D-lac = D-lactic acid produced
[4] - = absence of production or growth; + = production or growth; nd = not determined; sl = slow

Three of these were isolated about 1960 and 6 in 1972.

As MRS broth contains 0.5% citrate the isolates were tested for gas production both with and without citrate. When citrate was omitted 8 produced gas while 2 did not grow. Thus the 8 strains were truly heterofermentative on glucose. When the isolates were plated on citrate differentiating media (either McKay's or KCA) 4 showed citrate fermentation. As all grew at 15 C they did not belong to the thermophilic group of lactobacilli.

Table xxviii. Growth characteristics of various heterolactic lactobacillus cultures originally isolated from Cheddar cheeses.

Culture	Gas in MRS[1]		Growth in MRS at		Growth in[2]			% D-lac[3]	Colonies in[4]	
	+cit	-cit	15C	45C	M17	RSM	YM		K&M	KCA
2	+[5]	+	+	-	+	-	+sl	53	blue	+
4	+	+	+	-	+	-	+	54	"	+
5	+	+	+	+	+	-	+	45	"	+
6	+	+	+	+	-	-	+sl	77	"	+
22	+	+	+	-	-	-	+sl	31	nd	nd
24	+	-	+	-	-	-	-	0	white	-
25	+	-	+	-	-	-	-	41	"	-
26	+	+	+	-	-	-	-	26	"	-
27	+	+	+	-	-	-	-	27	nd	nd
30	+	+	+	-	-	-	-	29	nd	nd

[1] MRS = Man, Rogosa and Sharpe liquid medium, + cit = with normal citrate concentration, - cit = without citrate.
[2] Liquid media, M17 = medium of Terzaghi and Sandine (1975) with lactose as energy source, RSM = reconstituted skim milk, YM = RSM with 0.5% added yeast extract
[3] D-lac = D-lactic acid produced
[4] Agar media, K&M = medium of Kempler and M+cKay (1980), KCA = calcium citrate agar of Nickels and Leesment (1964)
[5] - = absence of production or growth; + = production or growth; nd = not determined; sl = slow

Isolate 2 was selected for use in cheese manufacture as it was able to utilize both lactose and citrate and clotted yeast-milk.

II.5.2.3 Pediococci. The characteristics of the pediococcus isolates are shown in Table xxix. Three isolates were from N.Z. made Cheddar cheeses from two different sources, and 4 from cheeses made in the U.S.A. All the cultures grew in MRS broth at 30 C producing DL lactic acid without gas, all grew at 15 C but none grew at 45 C. All grew in M17 broth (lactose) however none clotted milk at 30 C. When RSM was fortified with 0.5% yeast extract all the strains were able to produce a clot, however some required at least 3 days incubation.

Table xxix. Growth characteristics of various pediococcus cultures originally isolated from Cheddar cheeses.

Culture	Identity	Growth in MRS[1]			Growth in[2]			%D-lac[3]	ToptC[4]
		15C	45C	gas	M17	RSM	YM	in MRS	in YGM
3	ex TFF[5] (NZ)	+[6]	-	-	+	-	+	50	35.9
10	ex TFF (USA)	+	-	-	+	-	+	58	34.0
14	ex TFF (USA)	+	-	-	+	-	+	26	36.8
15	ex TFF (USA)	+	-	-	+	-	+	25	36.4
16	ex TFF (USA)	+	-	-	+	-	+	22	37.1
39	P5 (ex TDT[5])	+	-	-	+	-	+	53	35.0
40	P6 (ex TDT)	+	-	-	+	-	+	55	35.0

[1] MRS = Man, Rogosa and Sharpe liquid medium, gas = production of gas.
[2] Liguid media, M17 = medium of Terzaghi and Sandine (1975) with lactose as energy source, RSM = reconstituted skim milk, YM = RSM with 0.5% added yeast extract
[3] D-lac = D-lactic acid produced
[4] ToptC = Temperature optimum for growth
[5] TFF = Dr T.F.Fryer, NZDRI, TDT = Dr T.D.Thomas, NZDRI
[6] - = absence of production or growth; + = production or growth

The temperature optimum of each isolate was determined by incubation in yeast-glucose-milk for 18 h in a Toyo temperature gradient incubator. All had optimum temperatures between 34.0 and 37.1 C (mean 36.0, sd 1.1 C). Thus all the cultures appeared to be very similar and to exhibit the characteristics of <u>Pediococcus pentosaceus</u> since the temperature optimum of this species is 35 C (Kitahara, 1974).

Isolate 39 has been used previously for cheese manufacture (P5 of Thomas and Crow, 1983). It grew rapidly in the cheese when inoculated at 100 cfu/g from an MRS culture, reaching a peak of $5*10^7$ cfu/g at 7 days. This isolate rapidly interconverts D- and L-lactic acid to form a racemic mixture in both broth culture and in cheese.

<u>II.5.3. Growth Characteristics of NSLAB in Milk</u>. When Pe39 was inoculated into RSM from MRS culture, the count rose rapidly to reach $2*10^8$ cfu/g by 24 h (Fig. 15), with a culture pH of 5.8. Subculturing sequentially after 24h of growth yielded cultures peaking at counts of around $1*10^8$ cfu/g within 48 h. However, if the culture was held beyond the 24 h subculturing point, a low pH was obtained in only the first subculture from the MRS broth grown inoculum with that culture clotting in 3-4 days. Even after 7 days at 30 C, the pH of the fourth subculture in RSM had not dropped below 6.0.

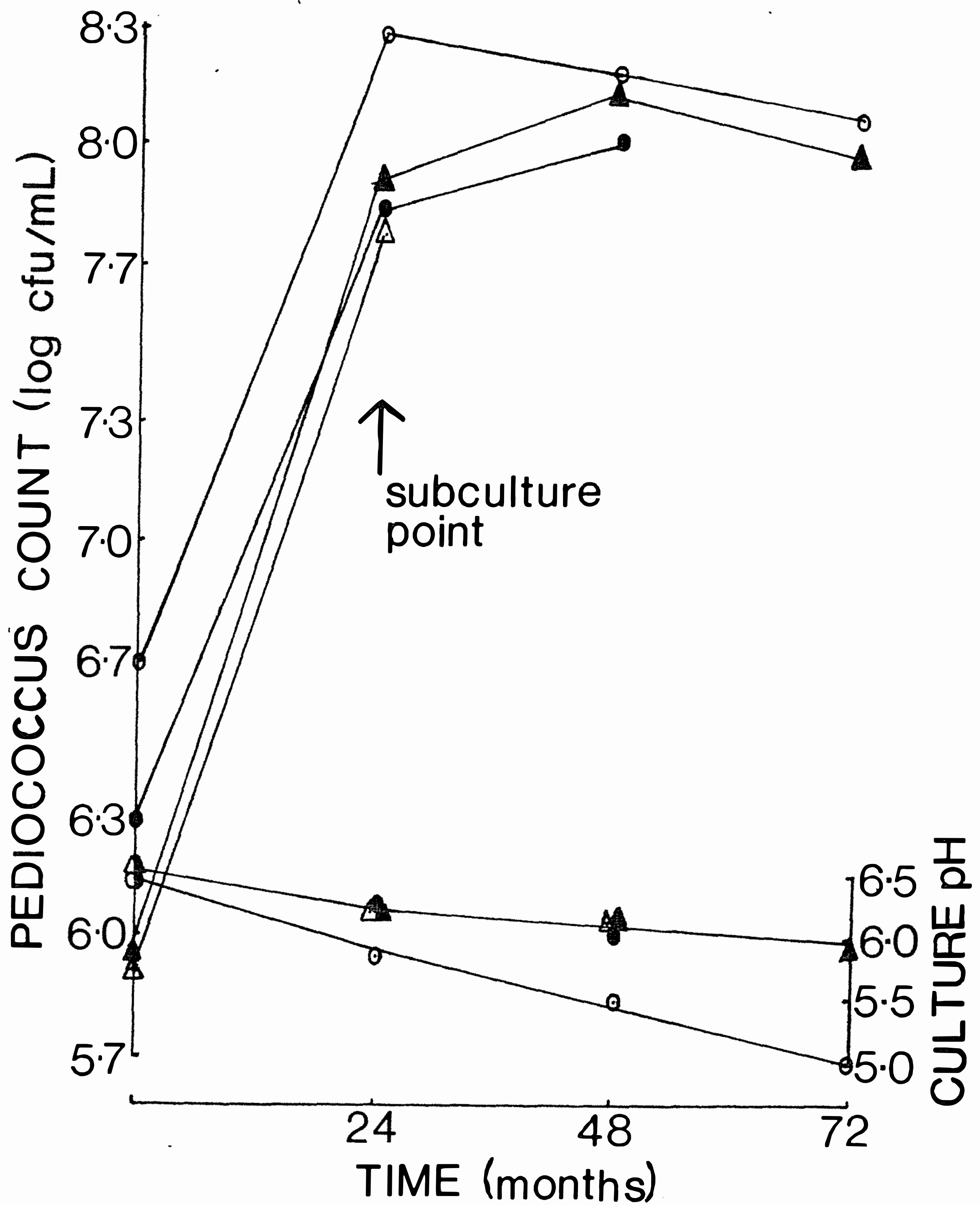


Figure 15. Growth and acid production of pediococcus isolate Pe39 in RSM. Pediococcus isolate Pe39 was inoculated from MRS broth into RSM, incubated at 30 C and subcultured (1% inoculum) every 24 hours. Growth was measured by plating onto MRS agar, and acid production by pH change. The first, second and fourth subcultures were incubated up to 48 hours beyond their subculture time at 24 hours. ⊙ - ⊙ First culture; ▲ - ▲ second culture; ● - ● third culture; △ - △ fourth culture.

The log mean counts after 48 h growth at 30 C in RSM of strain 2 (heterolactic lactobacillus), strain 43 (homolactic lactobacillus) and strain 39 (pediococcus) are shown in Table xxx. The heterolactic lactobacillus gave the highest counts ($2.3*10^8$ cfu/g), with both the pediococcus ($5.5*10^7$ cfu/g) and the homolactic lactobacillus ($4.0*10^7$ cfu/g) being substantially lower. For all 3 strains, the standard deviation was relatively low at around 50%.

In trials with Pe39, folic or folinic acid (0.001-0.1%) had no effect on pH or count in RSM culture. Addition of glucose plus tryptic digest of casein (glucose 1%, trypticase 0.5%), and addition of the extract of S. cremoris 584 lowered the pH but did not increase the count in RSM culture.

Addition of yeast extract increased the count to $1*10^9$ cfu/g and lowered the pH (Fig. 16).

II.5.4 Procedures for the Inoculation of NSLAB-free curd with NSLAB organisms. The method of growth of NSLAB for controlled addition to NSLAB-free cheeses in the aseptic vats, and the stage for their addition during manufacture, was investigated in the early phase of the project, before the use of aseptically-drawn milk. These experiments were carried out with bulk-herd tanker-delivered milk using Pe39 grown either in MRS broth or in RSM. Three stages in the manufacturing procedure were investigated - addition to the pasteurized milk

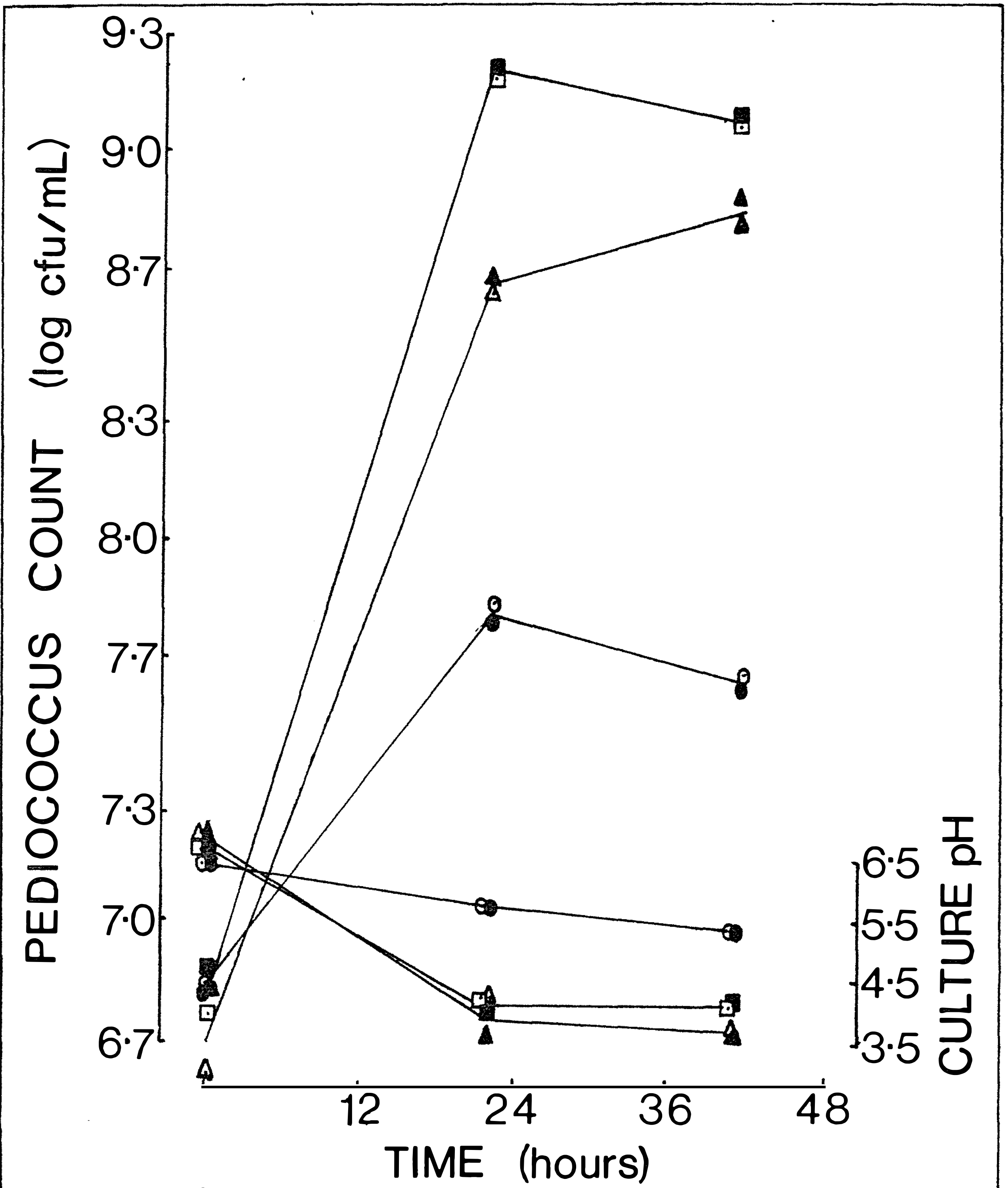


Figure 16. Growth and acid production of pediococcus isolates Pe3 and Pe39 in MRS broth, RSM and RSM plus 0.5% yeast extract. Pediococcus isolates Pe3 and Pe39 were inoculated from MRS broth into either MRS broth, RSM, or yeast milk and incubated at 30 C. Growth was measured by plating onto MRS agar, and acid production by pH change. □-■ MRS broth culture; ○-● RSM culture; △-▲ yeast milk culture. Open symbols, Pe3; closed symbols, Pe39.

Table xxx. Reproducibility of counts of NSLAB cultures grown for 48 h in RSM at 30 C.

Run No	Ho 43[1]	He 2[1]	Pe 39[1]
A	-[2]	$3.4*10^8$	-
B	-	$2.1*10^8$	-
C	$5.8*10^7$	-	-
D	-	-	$6.4*10^7$
E	-	-	$7.3*10^7$
F	$4.7*10^7$	$2.5*10^8$	-
G	-	$1.6*10^8$	-
H	$8.3*10^7$	-	$10.7*10^7$
I	-	-	$6.4*10^7$
J	-	$3.1*10^8$	-
K	$5.4*10^7$	$3.4*10^8$	-
L	$6.0*10^7$	-	$5.8*10^7$
M	-	-	$3.7*10^7$
N	$4.1*10^7$	$3.0*10^8$	-
O	-	$1.8*10^8$	-
P	-	$1.7*10^8$	-
Q	$3.3*10^7$	-	-
R	-	-	$5.1*10^7$
S	$2.5*10^7$	-	$4.2*10^7$
T	$5.2*10^7$	-	$5.8*10^7$
U	-	-	$4.3*10^7$
V	$3.1*10^7$	-	-
W	$4.8*10^7$	-	-
X	-	$1.3*10^8$	-
Y	-	-	$4.9*10^7$
Z	$1.5*10^7$	-	-
AA	$3.2*10^7$	-	$7.9*10^7$
AB	$2.9*10^7$	-	$4.8*10^7$
AC	$4.9*10^7$	-	$3.4*10^7$
AD	$3.0*10^7$	-	-
Number	16	10	15
Mean	$4.0*10^7$	$2.3*10^8$	$5.5*10^7$
+s.d.	$6.1*10^7$	$3.2*10^8$	$7.5*10^7$
-s.d.	$2.6*10^7$	$1.6*10^8$	$4.0*10^7$

[1] Counts (in cfu/mL) of the various cultures. Ho 43 = homolactic lactobacillus isolate 43, He 2 = heterolactic lactobacillus isolate 2, Pe 39 = pediococcus isolate 39.
[2] - = Not grown on that run. Each run was the inoculum culture used for cheese manufacture in Section II.5.5.

with the starter; addition to the curd immediately after the removal of the whey at running; and addition to the curd after milling and approx. 5 min before salt addition. The results are shown in Table xxxi.

Table xxxi. Effect of growth medium of NSLAB (Pe 39) and site of inoculation on the count in cheese.

	Inoculum Culture[1] (*10^-8)	Dry[2] (*10^-4)	Dress[2] (*10^-4)	1d[2] (*10^-5)	7d[2] (*10^-7)	14d[2] (*10^-8)
MRS Grown						
Control	-[3]	-	<.0003	.00001	.0000005	.000003
Milk	5.3	1.46	1.62	18.2	19.2	1.5
Dry	5.3	1.46	2.4	9.6	30.7	1.9
Mill	15.7	-	1.59	40.6	18.9	2.1
Mean (+NSLAB)		-	1.84	19.2	22.3	1.8
RSM Grown						
Control	-	-	-	0.009	-	0.000007
Milk	0.7	0.5	1.3	21.9	0.1	1.14
Dry	6.33	4.0	4.5	2.6	1.2	0.007
Mill	6.33	-	5.0	2.7	0.1	0.07
Mean (+NSLAB)		-	3.1	5.3	0.2	0.8

1 Bacterial counts in cfu/mL
2 Bacterial counts in cfu/g, d = days
3 - = not determined

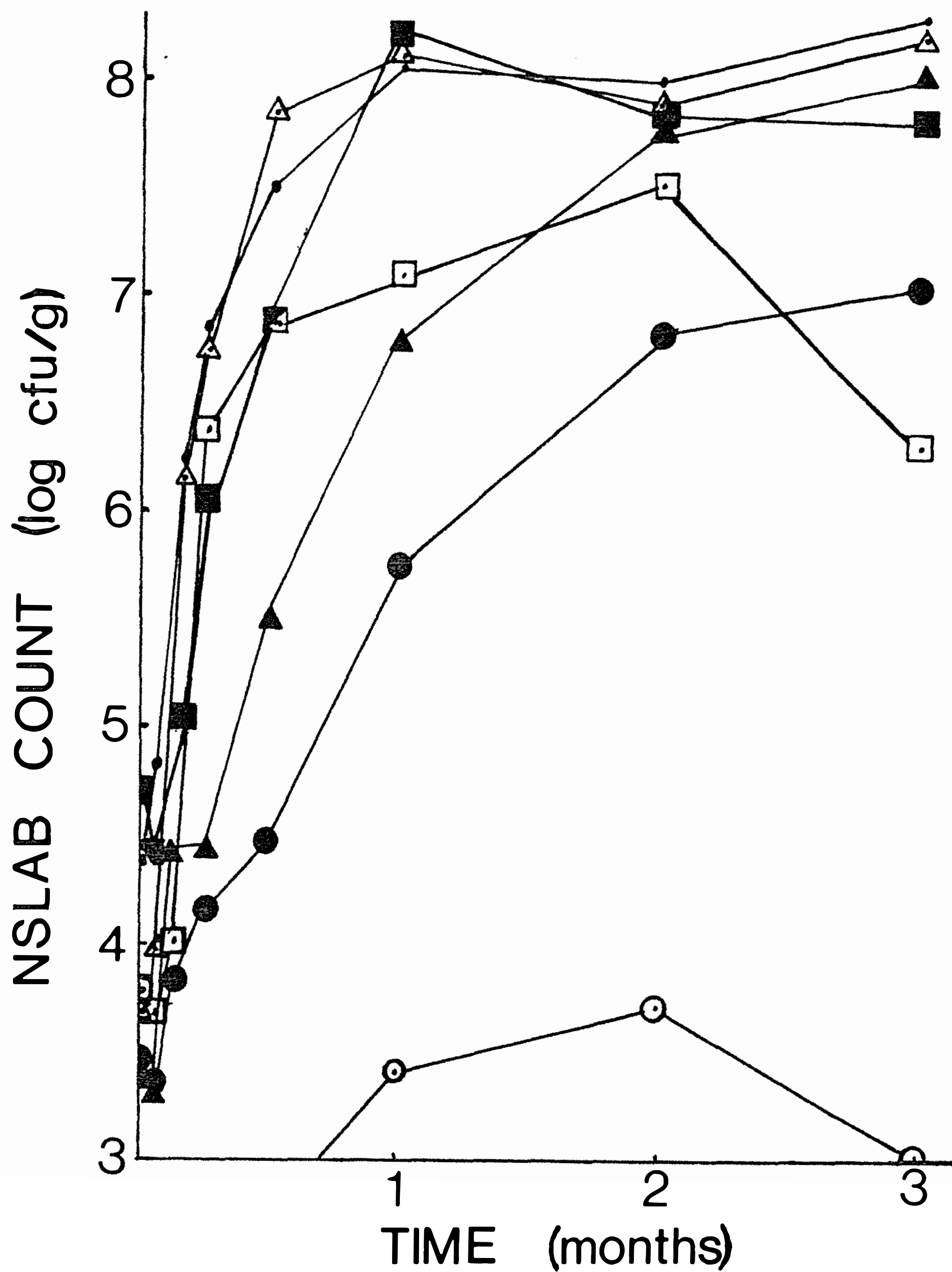
The MRS grown P39 grew rapidly attaining levels in excess of 10^8 cfu/g cheese in 7 days when inoculated at either of the three sites. The mean counts (in cfu/g) at four different times were: 5 min. after pressing started (dressing),

$4*10^4$; at 1 day, $4*10^6$; at 7 days $2*10^8$; and at 14 days, $2*10^8$. Growth in RSM culture resulted in lower counts so a higher inoculum volume was used to maintain the initial count of the bacteria. The rate of growth was slower than with the MRS grown cultures and the mean counts (in cfu/g) at the different times were: at dressing $3*10^4$; at 1 day, $5*10^4$; at 7 days $3*10^6$; and at 14 days, $8*10^7$. The count in the cheeses with the culture inoculated into the milk appeared to increase more rapidly than the count in the cheeses inoculated at either of the two sites.

Five NSLAB isolates selected for cheesemaking trials were tested to determine whether they grew in cheese under these inoculation conditions (Fig. 17). Two homolactic lactobacilli (isolates 44 and 45) grew more rapidly than did pediococcus 39, while two (isolates 38 and 43) grew more slowly. The heterolactic lactobacillus (isolate 2) appeared to grow rapidly initially but to plateau at a lower level of about 10^7 cfu/g.

<u>II.5.5 Growth of NSLAB Organisms Deliberately Added to Aseptically Manufactured Cheeses</u>.

<u>II.5.5.1 Initial Trial</u>. The growth of the three selected strains was further tested by inoculation into the milled curd of cheeses made aseptically from non-aseptically drawn milk,



<u>Figure 17. Growth of 6 NSLAB isolates in aseptically manufactured Cheddar cheeses</u>. Six NSLAB isolates were added at milling, to the curd of Cheddar cheeses made in the aseptic vats from pasteurized bulk-tank milk. The cheeses were matured at 12 C and growth of the NSLAB organisms was determined by plating samples of the cheese taken in the first three months of maturation onto LBS agar. ⊙-⊙ Control (no additions); ⊡-⊡ He 2; ■-■ Pe 39; ●-● Ho 38; ▲-▲ Ho 43; △-△ Ho 44; •-• Ho 45.

collected as described in Section I.A.2.2. The results are the means of four trials with each culture at both maturing temperatures.

The bacterial composition of the raw and pasteurized milks was reported in Section I.A.2.3, and the chemical composition of the cheeses in Section I.A.2.4.

Bacterial Counts in the Cheeses. The log mean counts of NSLAB in the cheeses (as computed by the analysis of variance) are presented in Fig 18. The pediococcus culture grew rapidly reaching a plateau of $3*10^7$ cfu/g by 35 days at both 10 and 15 C. The homolactic lactobacillus at 15 C also reached a plateau (of $5*10^7$ cfu/g) by 35 days but at 10 C the plateau took 3 months to be reached and was lower at $1*10^7$ cfu/g. The count in the cheese containing heterolactic lactobacillus 2 rose only about 1.5 logs in the first 6 months at 15 C and less than 1 log in 9 months at 10 C. From the data in Fig. 18, generation times were calculated (Table xxxii).

Table xxxii. Generation times (in days) for NSLAB deliberately added to cheese.

	Control		Pe 39[1]	He 2	Ho 43
	Initial	Later			
10 C	10	-[2]	2.1	-[3]	4.7
15 C	4	21	1.6	7.5	1.7

[1] Pe 39 = pediococcus isolate 39, He 2 = heterolactic lactobacillus isolate 2, Ho 43 = homolactic lactobacillus isolate 43
[2] no secondary slope
[3] no growth

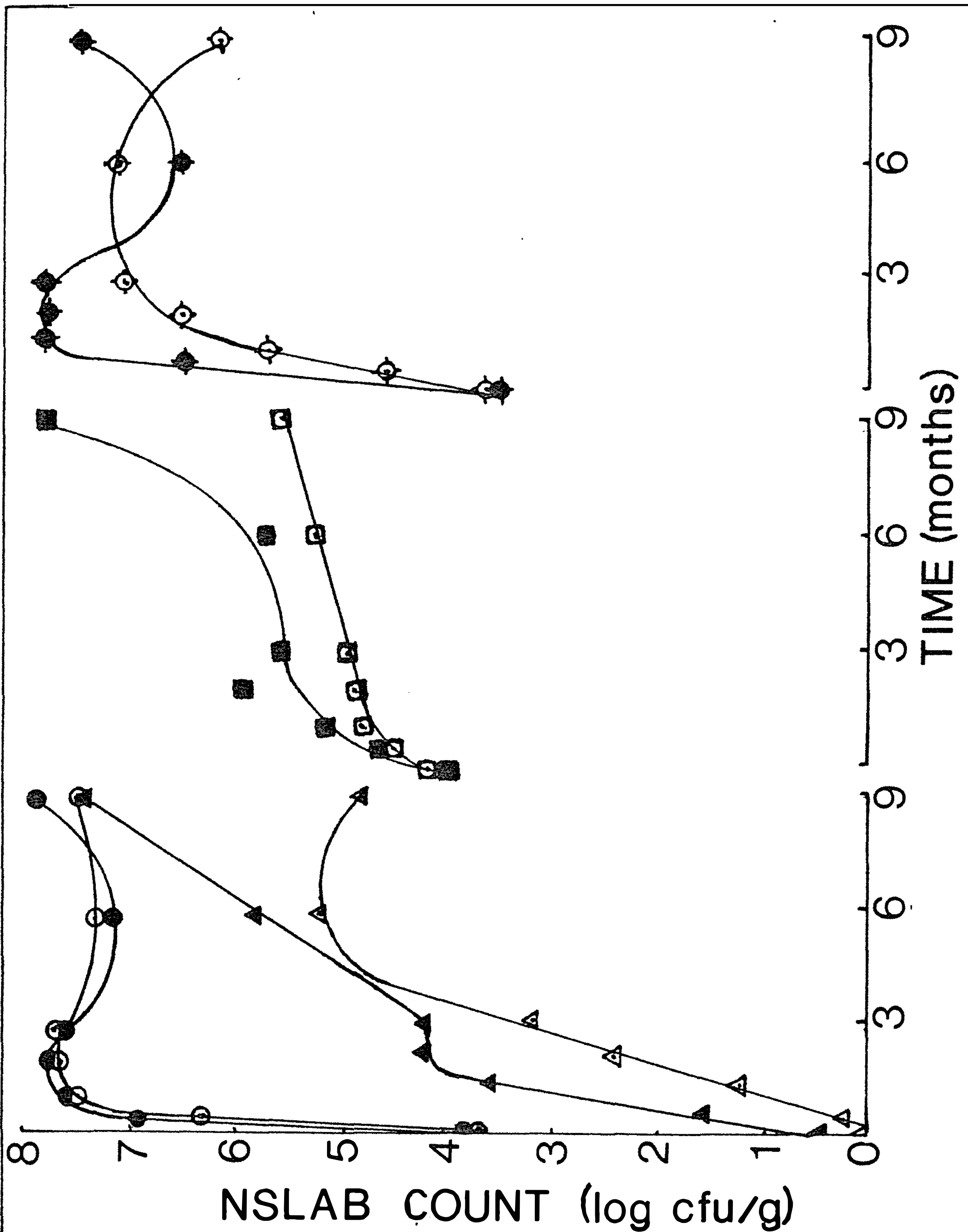


Figure 18. Growth of deliberately added NSLAB in aseptically manufactured cheeses. Three representative NSLAB isolates were added at milling, to Cheddar cheese curd made aseptically from milk not aseptically drawn. The growth of the NSLAB organisms was determined during maturation at either 10 C or 15 C. ⊙-● Pe39; ◘-■ He2; ⌖-◆ Ho43; △-▲ control (no added NSLAB). Open symbols, matured at 10 C, closed symbols, matured at 15 C.

II.5.5.2 The Effect of NSLAB Addition on Texture and Flavour.

II.5.5.2.1 Statistical Approach. Since the heterolactic lactobacillus did not grow rapidly in cheese, the full texture and flavour trial was restricted to only 2 NSLAB cultures - the pediococcus and the homolactic lactobacillus. A series of 12 cheese manufacturing trials was completed over a 1 month period in October and November 1985. Each vat was used to make one cheese which was cut in half when 1 day old. The two half cheeses were matured separately - one at 10 C and the other at 15 C. Samples were taken from each half cheese for bacterial, textural and flavour analysis. Since the cheeses held at 15 C developed excessive and unbalanced flavours by 6 months, the panel assessment of texture and flavour was terminated at that stage.

As cheese from one vat was split and matured at the two temperatures and the samples for each time analysis were from the same cheese, the data could be analysed as a true spilt plot design described by the Anova

Replicate,Treatment/ Temperature/ Time

However, since the cheeses were made over a short period in random order, the replicate effect could be considered as part of the error, so the data was analysed by the Anova

Treatment,(Rep<Trt)/ Temperature/ Time

The flavour panel data was treated with the subsamples not as error.

<u>II.5.5.2.2 Cheese Composition</u>. The composition of the cheeses is given in Table xxxiii. No temperature or time factors were involved and there was no significant effect of treatment.

As has been observed previously with cheeses made in the aseptic vats, the moisture content was higher than is normal for N.Z. Cheddar cheese, with a mean of 37.03% moisture and 55.66% MNFS. All other factors appeared to be within the normal range.

<u>Table xxxiii. Chemical composition of cheeses made in the aseptic vat with and without added NSLAB cultures</u>.

	Mean	Standard deviation
Moisture (%)	37.03	.80
Fat (%)	33.58	1.06
pH	5.25	.10
Salt (%)	1.87	.12
Calcium (mM/kg)	205.70	9.80
MNFS (%)	55.66	1.46
FDM (%)	53.14	1.81
SNF (%)	29.51	1.30
S/M (%)	5.05	.33
Ca/SNF (mM/kg)	697	18

<u>II.5.5.2.3 Bacterial Counts</u>. The counts of starter and nonstarter bacteria are shown in Figs. 19 and 20. In control cheeses the starter count decreased from $4*10^8$ to $1.5*10^8$ cfu/g in the first month (Fig 19). Thereafter, the count in the cheeses held at 10 C fell uniformly to reach levels of $5*10^4$ cfu/g at 9 months, while the count in the cheeses held

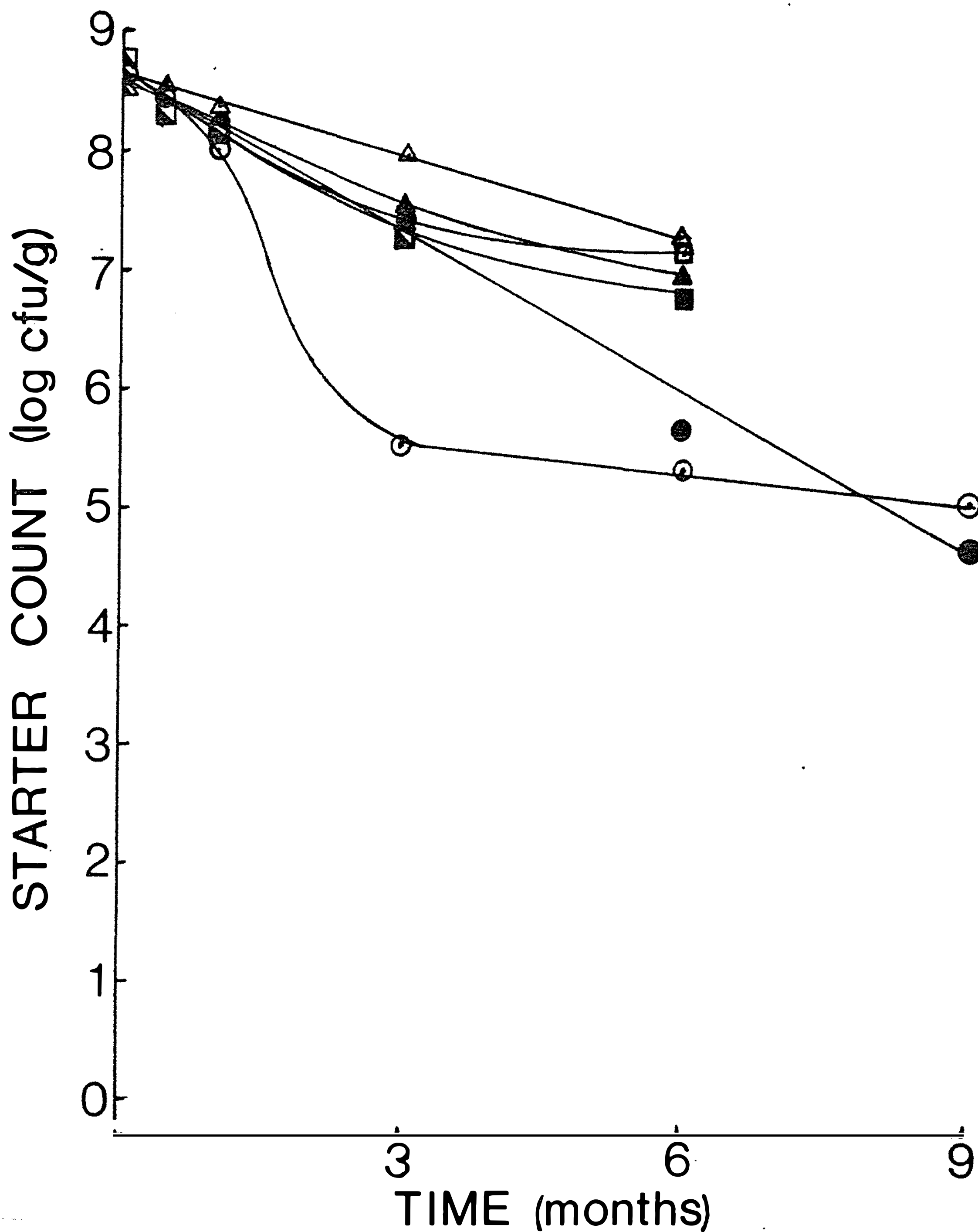


Figure 19. Starter counts in aseptically manufactured cheeses with deliberately added NSLAB. Starter counts were determined in cheeses made in the aseptic vats from milk drawn aseptically. The results are the mean values of four replicates of a 3*2 BIBD experiment of three NSLAB conditions (control plus two NSLAB cultures added to curd at milling), and the cheeses were matured at either 10 C or 15 C in barrier bags without waxing. ⊙-● Control cheeses (no added NSLAB); △-▲ Pe 39 added; ⊡-■ Ho 43 added. Open symbols, 10 C; closed symbols, 15 C.

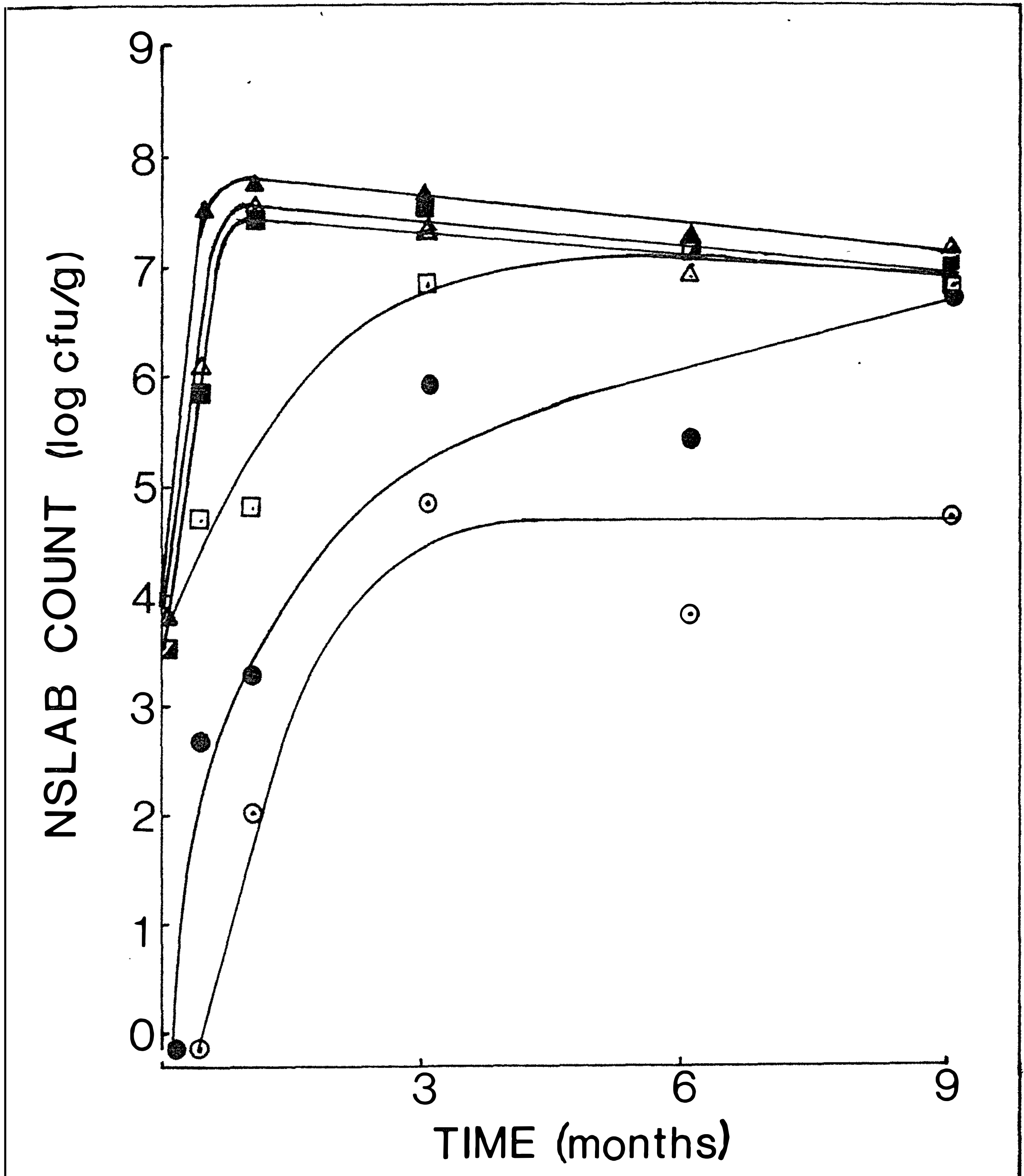


Figure 20. Counts of deliberately added NSLAB organisms in aseptically manufactured cheeses. NSLAB counts were determined in cheeses made in the aseptic vats from milk drawn aseptically. The results are the mean values of four replicates of a 3*2 BIBD experiment of three NSLAB conditions (control plus two NSLAB cultures added to curd at milling), and the cheeses were matured at either 10 C or 15 C in barrier bags without waxing. ⊙-● Control cheeses (no added NSLAB); △-▲ Pe 39 added; ⊡-■ Ho 43 added. Open symbols, 10 C; closed symbols, 15 C.

at 15 C fell rapidly to around $4*10^5$ cfu/g at 3 months, and then only slowly, reaching $1*10^5$ cfu/g at 9 months. The count on M17 agar of cheeses to which NSLAB had been added fell slowly to reach levels of $1*10^7$ cfu/g at 6 months. At this stage the counts were not of starter, but reflected the high levels of NSLAB in the cheeses.

No NSLAB were detected in control cheeses held at 10 C either at day 1 or 14 (Fig 20). Thereafter, the count rose to reach a plateau of around $4*10^4$ cfu/g at 3 months. No further increase in count was observed. In control cheeses at 15 C, the count rose rapidly after day 1, reaching levels around $1*10^5$ cfu/g at 3 months. Thereafter the rate of increase declined and the count at 9 months was $5*10^6$ cfu/g.

In the cheeses with added pediococci (Fig 20) the count rose rapidly from the 1 day count of $3*10^3$ cfu/g. At 15 C the count reached $3*10^7$ cfu/g by 14 days, peaked around $5*10^7$ cfu/g by 1 month, and then declined only slowly to be $1.5*10^7$ cfu/g at 9 months. At 10 C the count increased slightly more slowly but still peaked by 1 month at a level of $3*10^7$ cfu/g. By 9 months, the count was still around $1*10^7$ cfu/g. In the cheeses with added homolactic lactobacilli, the count at 1 day was $2.5*10^3$ cfu/g. In the cheeses at 15 C the count peaked by 1 month at a level of $2*10^7$ cfu/g and did not decrease. However, in the cheese held at 10 C, the count increased more slowly reaching levels of $5*10^6$ cfu/g at 3 months and peaking at around $1*10^7$ cfu/g by 6 months.

II.5.5.2.4 Texture and Flavour of Cheeses Held for 6 months at 10 and 15 C. The F-values and estimates of significance for the textural and flavour differences between the cheeses made with added pediococcus or homolactic lactobacillus are shown in Table xxxiv. The means of all the significantly different attributes are shown in Table xxxv.

Table xxxiv. F-values and estimates of significance of texture and flavour attributes of cheeses made in the aseptic vat with added NSLAB cultures and held for 6 months at 10 C and 15 C.

	trt[1]	Temp	trt*T	time	trt*t	T*time	trt*T*t
Firmness [2]							
Rubbery		***[3] 35.93		** 10.49			
Crumbly		*** 125.72					
Smooth		*** 59.64		** 10.52			
Sticky							
Mealy		* 7.73		*** 34.99			
Gritty		*** 23.89		* 6.33			
Acid/sour		*** 43.44					
Fruity		*** 155.60	*** 20.32	** 11.89			
Sulphide		*** 157.78	* 6.78	*** 24.49		** 11.35	
Sharp		*** 62.93		* 6.71		* 7.35	
Bitter		*** 26.20		* 6.91		** 6.77	* 4.54

[1] trt = treatment, Temp or T = temperature, t = time
[2] For clarity, the data for non-significant factors has been omitted
[3] * - P < 5%, ** = P < 1%, *** P < 0.1%

Table xxxv. Significant means of the differences in texture and flavour attributes of cheeses made in the aseptic vat with added NSLAB cultures and held for 6 months at 10 C and 15 C.

	Temperature		time	
	10 C	15 C	3 mo	6 mo
Firmness				
Rubbery	2.32	1.62	2.19	1.75
Crumbly	2.65	4.17		
Smooth	4.42	3.54	4.12	3.85
Sticky				
Mealy	2.25	2.67	2.89	2.05
Gritty	2.02	2.85		
Acid/sour	4.42	4.99		
Fruity	2.37	3.27	2.61	3.02
Sulphide	.59	1.63	.80	1.43
Sharp	1.16	1.91	1.35	1.72
Bitter	.29	.66	.38	.57

Texture. There were no differences in texture between the control, added pediococcus and added lactobacillus cheeses that could be attributed to the treatment. There were however differences due to the storage temperature and to time. Rubberiness and smoothness decreased with temperature while crumbliness, mealiness and grittiness increased. Rubberiness, smoothness and mealiness all decreased with time.

Flavour. There were no significant effects of treatment on the flavour in the cheeses, however all the flavour attributes were significantly increased (P<0.1%) by the storage temperature. Time increased fruitiness (P<1%), sulphide (P,0.1%), sharpness (P<5%) and bitterness (P<5%).

There were two significant treatment by temperature interactions (Table xxxvi) with the cheeses with added homolactic lactobacillus having the highest scores for both fruitiness (P<0.1%) and sulphide (P<5%). There were also three significant temperature by time interactions - for sulphide, sharpness and bitterness with P<1%, P<5% and P<1% respectively.

Table xxxvi. Interaction means of the differences in texture and flavour attributes of cheeses made in the aseptic vat with added NSLAB cultures and held for 6 months at 10 C and 15 C.

Treatment*Temperature

	Fruitiness		Sulphide	
	10 C	15 C	10 C	15 C
Control	2.27	2.59	.51	1.34
Homo	2.36	3.80	.64	2.09
Pedio	2.48	3.39	.63	1.44

Temperature*Time

	Sulphide		Sharp		Bitterness	
	3 mo	6 mo	3 mo	6 mo	3 mo	6 mo
10 C	.49	.69	1.16	1.15	.30	.29
15 C	.10	2.15	1.54	2.27	.46	.84

In all three cases, the intensity increased more rapidly in cheeses held at 15 C than in cheeses held at 10 C.

There was a significant three-way interaction for bitterness (P<5%) showing no change at 10 C but a significant increase in bitterness in the cheese with added lactobacillus stored at 15

C (Table xxxvii). While the threeway interactions for fruitiness, sulphide and sharpness were not significant (Table xxxiv) their means (Table xxxvii) show that for fruitiness and sulphide the greatest increase in intensity was in the cheeses with added homolactic lactobacillus stored at 15 C. For sharpness the cheeses with added pediococcus stored at 15 C had the greatest increase in intensity.

II.5.6 Conclusions. Inspection of the NSLAB isolates in the NZDRI culture collection show that *L. plantarum* was the most frequently isolated species held, with *L. casei*, heterolactic lactobacilli and pediococci all being isolated approximately as frequently.

Table xxxvii. Threeway interactions for the differences in texture and flavour attributes of cheeses made in the aseptic and open vats and held for 6 months at 10 C and 15 C.

	Friutiness		Sulphide		Sharp		Bitterness	
	3 mo	6 mo	3 mo	6 mo	3 mo	6 mo	3 mo	6 mo
10 C								
Control	2.03	2.50	.52	.50	1.15	1.10	.18	.29
Homo	2.42	2.30	.45	.82	1.21	1.21	.45	.24
Pedio	2.34	2.61	.50	.76	1.13	1.24	.25	.33
15 C								
Control	2.24	2.94	.70	1.97	1.39	2.06	.30	.62
Homo	3.45	4.14	1.52	2.66	1.73	2.14	.55	1.31
Pedio	3.19	3.58	1.09	1.79	1.50	2.64	.53	.58

The inoculation of RSM grown cultures into cheese curd at milling gave a controlled rate of growth of both the homolactic lactobacillus and pediococcus. Despite the implication of heterolactic NSLAB in off-flavour production in Cheddar cheese cooled at very slow rates (Fryer, pers.comm.), the strain tested did not grow significantly at either 10 or 15 C. Since higher temperatures would not be recommended commercially, and this project was aimed at the production of acceptable Cheddar cheese flavour at normal temperatures, investigations using the heterolactic lactobacillus were discontinued.

The addition of NSLAB had no detectable effect on the cheese texture. Neither was there any effect on the flavour of cheeses held at 10 C. However, in the cheeses held at 15 C, NSLAB addition (especially the homolactic lactobacillus) resulted in cheeses with significantly increased bitterness at 6 months. While not statistically significant, the fruitiness, sharpness and sulphide intensities were highest in the cheeses with NSLAB addition. This effect was similar to the effect noticed with adventitious NSLAB, in Sections II.3 and II.4. Since the effect was so small, the trial was not repeated with the cheese surface waxed to prevent contamination.

II.6 Discussion and Conclusions for Parts I and II.

The aim of the current research was to determine the effect of NSLAB on the development of texture and flavour of Cheddar cheese matured in impermeable cheese bags. The strategy was to eliminate the adventitious NSLAB flora by manufacturing the cheese in enclosed aseptic cheesemaking vats. Selected, representative strains also reintroduced into aseptically made cheese curd.

II.6.1 Manufacturing Conditions Required to Produce NSLAB-free Cheeses.

The manufacture of bacteria-free cheeses appears to be a difficult and expensive operation. Kleter (1976) obtained sterile cheeses by a combination of strict preparation of the cows before milking, pasteurization of the milk, and the use of autoclaved vats. Others (Le Bars et al, 1975, O'Keefe et al, 1976a,b) required the presence of antibiotics to ensure sterility. However, simple precautions may be sufficient to ensure NSLAB-free cheeses since various workers have produced such cheeses without these stringent precautions (Chapman et al, 1966; Perry and McGillivray, 1964, and Lowrie et al, 1974).

The system described in this report was designed to be only as complex as needed. Simple precautions in the milking of the cows were sufficient to ensure no NSLAB contamination of the milk (Section I.A.4). This was required since at least

some NSLAB organisms were capable of surviving pasteurization at significant levels (Section I.A.3). Only when complete sterility is required (as in the production of starter-free cheeses) would the more stringent precautions of Kleter and Vries (1975) be required.

The vats were made of materials which were only required to withstand temperatures of 100 C, since, as no large autoclave was available autoclaving (as was done by Kleter, 1975; Chapman *et al*, 1966, Law *et al*, 1976a,b) was not possible. Soaking the vats in Iodophor solution overnight followed by steaming for one hour was sufficient to ensure the absence of NSLAB when the vats were swabbed and when sterile UHT milk was incubated in the vats (Section I.A.1.5). This confirms the previous reports of work conducted in chemical and/or steam sterilized vats (Perry and McGillivray, 1964; Lowrie *et al*, 1974).

Some reports (for instance, Perry and McGillivray, 1964; Lowrie *et al*, 1974) indicate that it is possible to manufacture NSLAB-free cheeses from milk which was not aseptically drawn. The thermal death characteristics of NSLAB (Section I.3) suggest that this will be possible only so long as the NSLAB population of the raw milk does not contain *L. casei* or *Leu. lactis* organisms, and that the total population of NSLAB is low. Therefore, since simple precautions in the milking and transport of the milk were adequate to ensure that NSLAB were excluded from the raw milk (and also significantly

reduced the total count of the pasteurized milk), these precautions are to be recommended for the additional security that they provide.

II.6.2 The Effect of NSLAB on the Maturation of Cheddar Cheeses. Previous attempts to introduce NSLAB into aseptically made cheeses have resulted in cheeses of poor flavour. This was particularly noticeable in the work with "reference floras" at the NIRD (Reiter *et al*, 1967; Fryer, 1968). Cheeses made without NSLAB have been reported to develop flavour as well as or more slowly than controls (Lawrence 1966).

The results of the present investigation show that when cheeses are matured at 10 C, there is no detectable difference in flavour whether or not NSLAB organisms are present (Section II.4). However, in cheeses matured for 6 months at 15 C, NSLAB-free cheeses were lower in both sharpness and sulphide attributes than cheeses with adventitious NSLAB present (Section II.4).

The increase in the sharpness was quite small, and the cheeses were very mild (a score of 2 on a scale of 10) indicating that sharpness was at the threshold of detection. This may be due to the starter used, since the pair 584 plus 134 have been used extensively in the New Zealand cheese industry to produce bland cheese, especially for the Japanese market.

The increase in the sulphide attribute in cheeses containing NSLAB was particularly noticable in cheeses matured in bags (II.4). It is likely that this is due, not to a greater rate of formation of sulphur compounds, but to their greater retention by the bag. Effectively, the waxed cheeses could be expected to loose a greater proportion of the sulphides produced. An alternative argument would be that the bagged cheeses had a lower redox potential which promoted degradation of sulphur containing amino acids. However the proponents of the redox potential argument have argued that the NSLAB maintain a low redox potential in cheese after starter death. If this were true, then in the non-bagged cheeses containing NSLAB there should have been a greater sulphide note than in the equivalent cheeses without NSLAB. No such difference was noted.

Since it is inherently difficult to obtain accurate redox potential measurements in cheese over the extended maturing periods involved, it may yet be a while until we can be sure whether waxed cheeses have a higher redox potential than bagged cheeses or cheeses containing NSLAB than NSLAB-free cheeses. It is obvious however, that the presence of NSLAB may increase the sulphide attribute in the cheeses. Most likely, the NSLAB contribute a greater resevoir of enzymes catalysing the reactions forming sulphide compounds.

Thus, starter alone appear to be quite capable of producing the environment required to produce the flavour associated with mild Cheddar cheese. They appear to be able to do this

without the intervention of NSLAB organisms. However, the NSLAB appear to contribute a greater ability to produce the sulphurous compounds required in very mature cheese but undesirable in young or mild cheese. The restriction of NSLAB counts in young cheese would not appear to be the cause of a reduction in the flavour of the mature cheese. Rather the reduction in mature flavours results from holding the cheese at too low a temperature during the remainder of the maturing process. As was shown by Fryer (1982), once the NSLAB flora was controlled by the rapid cooling, reducing the potential for heterolactic lactobacilli to dominate the flora at an early age, Cheddar cheese can be ripened quite successfully at rates dependent on the storage temperature. For cheese required to be mature, tasty, vintage or epicure, raising the temperature will increase the rate at which the NSLAB grow, the maximum population of the NSLAB and the rate of maturing both of sharpness and of the sulphide character.

It follows that the starter holds a central role in the maturing of cheese, a role which has been underestimated in the recent past as workers have striven to understand the postulated contribution of other bacteria. Starter commence by converting the lactose of the milk into lactate with the consequent development of both a reducing environment and a low water activity. Removal of lactose removes the most available energy source for the growth of other bacteria. The presence of a low redox potential further reduces the likelihood that contaminating bacteria can grow, while the low

water activity, manipulated by the control of the cheese composition, ensures that any bacteria which are able to grow, do so only relatively slowly. Apparently, only the NSLAB are adapted to rapid growth under these conditions.

The more subtle, but no less important role of the starter is to produce those enzymes which break down the cheese components to produce the flavour compounds. Among these are the caseins, the fat, and the fat globule membrane. The rate of these reactions will depend on at least four factors: i) the concentration on the catalysts (enzymes); ii) the temperature for the reaction; iii) the water activity and iv) the redox potential.

Many techniques have been used to increase the concentration of the catalysts. Obviously to increase the concentration of cells will increase the concentration of their enzymes. This approach was apparently sucessful (Lawrence and Gilles, 1967) although more recent attempts to reproduce this effect in cheese where the bacterial counts were actually measured, could detect neither differences in the starter count after cooking nor differences in flavour at maturity (K.W.Turner, unpublished results). The presence of too many cells in the cheese may lead to the development of undesirable bitterness (Lowrie et al, 1974). However, addition of a population of cells which cannot grow in the curd for one of a number of reasons (heat shocked, deficient in protease, or through

having a high temperature optimum for growth) has been promoted as a means of increasing the rate of maturation in cheese.

Another intriguing line of approach has been to add enzymes without the cells or microencapsulated enzymes to ensure their entrapment in the curd and later slow release (Law and King, 1985; Kirby and Law, 1987).

However in the midst of all this, one potentially very profitable area of research has been overlooked- the selection of the starter itself for its contribution to flavour development. Since the starter is the predominant (indeed almost exclusive) contributor of those compounds which produce cheese flavour, it follows that the differences in flavour which have been noted are due to the starter. The starters therefore vary in their complement of those enzymes which are required. Deliberate selection for fast flavour development would produce starter cultures which produce faster maturing cheese.

The recognition of strains of starter which produce flavour rapidly would lead to the possibility of comparing high and low flavour producing starters to discover how their enzyme complements differ. Since this is difficult in the laboratory where broth growth of the cultures raises the possibility that the production of the critical enzymes has been repressed, this line of research has not been pursued. It is time to review this approach since the cheapest, and commercially most acceptable way of introducing additional enzymes to cheese is

still their production in situ i.e. by the starter. Not only does this approach reduce ingredient costs, but it also obviates the need for the declaration of added enzyme preparations.

II.6.3 Pointers for Further Research. The research described here indicates that the manufacture of NSLAB-free cheese need not be as difficult as may have been expected, as long as suitable precautions are taken. Therefore projects involving such manufacture may be more readily undertaken.

Starter contain the capability to produce Cheddar cheese flavour, however further research needs to be directed to determining which strains produce the flavour compounds most readily, and what biochemical reactions have been promoted. Development of new strains should then concentrate on promoting those attributes. Since NSLAB when kept under control exert little effect on the early generation of flavour, this work need not initially involve aseptic manufacture of the cheese.

As NSLAB cultures obviously contain the ability to produce some potentially desirable flavour components, further research is required to identify which strains have the greatest benefit with the fewest side effects. Deliberate addition of selected strains to cheese need not require the use of aseptic manufacture since the added bacteria should

outgrow the adventitious flora if care is taken. However, it is important that the inoculum cultures are grown in sterile reconstituted milk, not in broth culture.

The role of a low redox potential developed by the starter in promoting the reactions of maturation is still not well researched. Aseptic manufacture, with or without the addition of selected strains of NSLAB, could be used to elucidate this effect. However, meaningful results will require the inventive use of partially permeable cheese bags admitting oxygen at different rates, and the attaching of the redox electrodes securely through the bags in such a manner that the electrode never looses contact with the cheese throughout the life of the experiment.

Part III. THE GROWTH OF LEUCONOSTOC ORGANISMS IN CHEESE

The starter cultures used to make most cheese except Cheddar cheese are composed of mixtures of *Streptococcus cremoris* and *S. lactis* along with *S. diacetylactis* and leuconostoc strains. While it is possible to enumerate leuconostocs grown in the presence of starter organisms by the use of a selective inhibitor, there has been no system devised to date which can selectively enumerate leuconostoc strains grown in the presence of NSLAB. Since all cheese made in open vats develop a flora of NSLAB, it is difficult to follow the growth or death of starter leuconostocs beyond the first few days of maturing.

Therefore, it was decided to attempt to manufacture cheese without NSLAB contamination using starter cultures which contained leuconostoc organisms. By this means, it should be possible to follow the growth and death cycle of the leuconostocs and also determine their effect on the citrate content of the cheese.

Part III describes the results of this investigation.

III.1. Literature Review.

Gas producing coccus-like organisms similar to those now called leuconostoc were reported in cheese by Evans in 1918. Since that time, many reports have appeared linking the presence of leuconostoc organisms in the starter culture with the production of enhanced flavours and eyes in a wide range of cheese. Much of the early work was reviewed by Fryer (1969).

Description of the Genus Leuconostoc. Leuconostocs form a somewhat ambiguous genus within the Family Streptococcaceae, characterized by the heterolactic fermentation of glucose with the production of ethanol, acetate, D-lactate and CO_2. Recent investigations of DNA homology (Garvie, 1976; Hontebeyrie and Gasser, 1977; Garvie, 1981,1983) and enzyme immunological specificity (Gasser and Gasser, 1971; Hontebeyrie and Gasser, 1975; Gasser and Hontebeyrie, 1977) have established that three of the originally recognised species (*Leu. mesenteroides*, *Leu. dextranicum* and *Leu. cremoris*) are closely enough related to be considered subspecies of *Leu. mesenteroides* (Garvie,1983). Their key physiological features of interest to dairy technologists are the ability to ferment glucose, galactose and lactose (some species 10 - 90% positive) and to metabolize citrate (subspecies *cremoris*, >90% positive, subspecies *mesenteroides* and *dextranicum* 10 - 90% positive) (Garvie, 1974).

Leuconostocs in Dairy Environments. The common habitat for leuconostocs is decaying plant material (Garvie, 1974), however all species except *Leu. oenos* have been reported from dairy environments. Perry and Sharpe (1959) reported the presence of a *Leu. mesenteroides* organism in the milk of a single herd, however they were unable to isolate the organism from cheeses made of that milk. Hucker and Pederson (1930) isolated *Leu. mesenteroides* and *Leu. dextranicum* but not *Leu. cremoris* from cheese.

The most common dairy sources are the starter cultures used in the manufacture of various cheeses and of lactic butter. *Leu. cremoris* is the most commonly reported species in this niche, however the other species have also been reported. These organisms are usually considered to be the causative organisms of aroma and eye formation in Dutch and other brine salted cheese varieties. In mixed culture starters, they are almost always found in the presence of *S. diacetylactis* which is also capable of forming gas and aroma. Cheddar cheese made with a starter containing leuconostocs developed slit defect (Sherwood, 1939a) while the defect was minimized in cheese made without leuconostocs in the starter. Leuconostocs were found in a Cheddar cheese with slit defect (Overcast and Albrecht, 1952). In cheese with only a *S. lactis* starter little openness was noticed, while addition of a leuconostoc culture with this starter caused distinct openness (Prouty and Goulding, 1946).

Metabolic Role of Leuconostocs in Cheese. The normally accepted role for leuconostocs in cheese (in common with S. diacetylactis) is to metabolize the citrate present in the milk with the formation of CO_2 gas. This fermentation produces acetate plus pyruvate, and since no NAD is used in its production, the pyruvate can be metabolized to flavourful compounds instead of lactate. Diacetyl, since it has an extremely low flavour threshold, is often the most noticable of these compounds.

Fryer (1969) concluded that the mode of citrate breakdown is identical to that of S. diacetylactis. Collins and Speckman (1974) observed a stimulation of the production of acetoin and diacetyl by Leu. cremoris when acetaldehyde was present in the media. Using radioactive acetaldehyde, they showed that the label appeared in the ethanol produced, not in the acetate, acetoin or diacetyl. In cheese, acetaldehyde may be present, especially if thermophilic cultures are used.

With S. diacetylactis, citrate is used only when a fermentable carbohydrate is present (Deibel and Seeley, 1974). However, there is some evidence (Mizuno and Jezeski, 1959) that leuconostoc species can utilize citrate in the absence of glucose at low pH. Whether this occurs in cheese is not known.

Since both S. diacetylactis and Leu. cremoris are added with the starter during cheesemaking, their numbers in the maturing cheese are likely to be high at first. However NSLAB organisms have also been shown to utilize citrate under

aerobic conditions (Thomas, 1986, 1987a). Thus, to establish that ongoing citrate utilization is due to the starter organisms, it is necessary to conduct experiments in the absence of NSLAB.

It was therefore decided to endeavour to manufacture cheese containing leuconostoc organisms in the aseptic vats, and determine their growth and death rates, while also measuring the rates of disappearence of citrate. Two different leuconostoc isolates were obtained from commercial cheese starters and their characteristics partially characterized (Section III.3.1 and III.3.2). The performance of these strains when inoculated into cheese was then determined in comparison to the performance of the type-strain of *Leu. cremoris* (Section III.3.3).

III.2 Methods.

III.2.1 Microbiological Methods.

III.2.1.1 Culture History and Maintenance. Type strains of leuconostocs were obtained from the NCDO collection. Culture Lc60 was isolated at the NZDRI and supplied by Mr. H.A.Heap. Culture Lc83 was isolated from a mixed strain starter culture (H83, Chr. Hansen Labs., Copenhagen, Denmark) plated on MRS agar containing tetracycline (see below). All strains were stored frozen at -75 C partially grown in MRS broth. Working cultures were maintained by weekly growth for 18 h at 30 C and storage of the freshly inoculated media at 4 C. RSM cultures were inoculated from the MRS cultures and maintained by weekly growth for 40 h at 30 C in RSM and storage of the freshly inoculated culture at 4 C.

III.2.1.2 Colony Counting. Colony counts of pure cultures of leuconostoc organisms were obtained by pour-plating serial dilutions in half-strength Ringers diluent in MRS agar (Difco Inc., after Man et al, 1960) with anaerobic incubation at 30 C for 48 h.

For selective plating of leuconostocs in the presence of starter organisms, 0.25 ug/mL tetracycline (Sigma Chemical Co., St. Louis, Miss. USA) was added to the MRS agar immediately before pouring the plates. Plates were incubated anaerobically at 30 C for 48 to 72 h. The level of tetracycline added was sufficient to inhibit the starter

organisms (S. cremoris 584 and 134) but did not inhibit the leuconostocs or the NSLAB organisms normally found in cheese (L.E.Pearce, pers. comm.).

Methods for preparing cheese samples for plating, and the methods for the determination of starter, NSLAB and other bacteria were as described previously (Sections II.2.5 and II.2.6).

III.2.1.3 Temperature Optimum Determination. The method for the determination of the temperature optimum for growth was as detailed in Section II.2.2.

III.2.1.4 Growth Sugar Determinations. The ability of leuconostoc cultures to grow on various sugars was determined by growth in MRS made from the constituent dry ingredients (BBL Microbiology Systems, Cockeysville, MD, USA, for microbiologicals, and BDH, Poole, England for Analar chemicals),which also contained 0.75% agar (Davis Agar, Christchurch, N.Z.). The sterile medium was dispensed into screw-topped tubes (Kimax, Kimble Products, Ohio, U.S.A) and the required volume of filter-sterilized sugar (Millipore Corp. Mass., U.S.A., 0.2 um pore size) added. The inoculum (diluted to give approximately 50 cfu/10 mL of agar medium) was added to the medium at 45 C and the agar rapidly solidified by cooling with water. After setting, the agar was topped with a plug of pure 1.5% agar. The agar cultures were

incubated at 10, 15, 22 and 30 C for up to 2 months, and the presence of colonies monitored against inoculated control tubes without added sugar.

III.2.1.5 Growth in RSM. The rate of growth of leuconostoc cultures in RSM was determined by inoculating 100 mL of RSM pre-equilibrated to 30 C, with a suitable serial dilution of RSM-grown cells, followed by incubation at 30 C in a water-bath. The cell density was measured by plating dilutions of the culture at various times into MRS agar as described in Section III.2.1.2.

III.2.2 Chemical Analyses. The methods used for routine chemical analysis of the cheeses were detailed in Section II.2.7.

Lactate Analysis. The concentrations of the individual L and D isomers of lactic acid produced by the cultures growing in RSM were determined enzymatically (Turner and Thomas, 1980).

Citrate analysis. The concentration of citric acid in broth or cheese was measured by the UV spectrophotometric method of Dagley (1974). Before analysis, 1 g samples of grated cheese were diluted to 10 g with Milli-Q water and homogenized at 20,000 rpm and 60 C using an Ultra-Turrax (Janke and Kunkel GmbH, Staufen, West Germany). The homogenized solutions were clarified by centrifugation for 10 min at 10,000 rpm and the

clear, defatted supernatent analysed. Where samples required storage before analysis, either the grated cheese sample, or the clarified supernatent was frozen at -18 C.

III.2.3 Citrate Utilization Studies. The ability of isolate Lc83 to utilize citrate in the presence or absence of a fermentable carbohydrate was determined using cells grown in MRS broth.

Cell Preparation. One hundred mL quantities of MRS broth were grown to full growth ($1*10^9$ cfu/mL) and harvested by centrifugation (10,000 rpm/10 min). These cells were washed twice with equal volumes of 2-(N-morpholino)ethane-sulphonic acid (MES) buffer (50mM, containing 50mM NaCl, and 10mM $MgCl_2$) at 4 C. The washed cells were resuspended in 20 mL of the same buffer and held at 4 C until use.

Reaction Conditions. The reaction flasks contained 5 mL of washed cell suspension added to 20 mL of MES buffer (50mM) containing 50mM NaCl, 10mM $MgCl_2$ and 3mM citrate at 30 C. One series of flasks was adjusted to pH 6.25 and the other to 5.25 with HCl. The reaction was initiated by the addition of the cells to the pre-equilibrated buffer. Control flasks were incubated without further adjustment, while 100 uL volumes of glucose (250mM) was added to the experimental flasks at 15 min intervals, immediately after the removal of 500 uL samples for citrate analysis. The samples for analysis were centrifuged immediately at 4 C and the supernatant frozen for later analysis.

III.2.4 Aseptic Vat Cheesemaking. The cheesemaking programme is outlined in Table xxxviii. All the procedures used for the aseptic manufacture of the cheese were as described previously (Section I.A.1) except in the following details.

The starter was reduced to 1.5% (2:1 584:134 by volume) and 0.12% of leuconostoc culture added at setting. The leuconostoc culture was grown in RSM for 24 h at 30 C, reaching levels of 10^7 to 10^8 cfu/mL without clotting the milk.

After inoculating, setting, coagulating and cutting as normal, the curds and whey were held at 33 C and gently stirred for 20 min before 40% of the volume was removed as whey and 20% of the initial volume was returned as sterile water (autoclaved 121 C for 15 min). The water was pumped from the sterilization tanks into the vats by filtered air pressure through a tube with a syringe wired to the end to enable it to be injected through the Suba seal. Filling time was 20 min.

Immediately after the whey was removed the cook was started, heating the vat contents to 38 C in 40 min. At 2h 50 min from setting the whey was all removed and the curd intermittently stirred by hand to prevent cheddaring. The curd was hooped 4h 00 min after setting. For dry salted cheeses, the salt was added 10 min before hooping.

Table xxxviii. Cheesemaking protocol for Gouda cheesemaking in the aseptic vats.

Time(h.min)	Temperature(C)	Comments
0.00	33.0	Initiate equilibrium
0.25	33.0	Add starter
0.30	33.0	Add rennet,cease stirring
1.10	33.0	Cut coagulum, take whey sample, start agitation
1.30	33.0	Remove 40% of volume as whey
1.35	33.0	Commence addition of sterile water to 80% of initial volume Commence heating
1.55	35.5	Water addition complete
2.10	38.0	Heating complete
2.50	37.0	Commence running whey, take whey sample
2.55	37.0	Whey removal complete Dry stir, take whey sample at outlet
3.50	34.0	Take pH sample, add salt if required
4.00	34.0	Commence hooping

After hooping (using the '10 lb.' hoops described previously - see Section II.2.3.2) the cheese were pressed at a pressure of 40 kPa using a 150 mm ram, for 1 h 30 min and then held overnight in a 15 C room. Following dehooping and sampling on the next morning all the cheese were bagged into cheesebags, except for one of the nonsalted cheese which was immersed for 36 h in autoclaved brine solution (23% NaCl at pH 4.8, containing 0.2% calcium as $CaCl_2$) in a sterile stainless steel beaker. All cheeses were held at 10 C in a curing room until the end of the brining period. After brining was complete, all the cheeses were wax coated by dipping into hot wax (II.2.3.4), vacuum sealed in cheesebags and placed in the

curing rooms. One of the dry salted cheeses was held at 10 C while the other dry salted cheese, the nonsalted cheese and the brined cheeses were held at 15 C.

III.3 Results.

III.3.1 Characterization of Leuconostoc Isolates.

Growth in Media Containing Tetracycline. Both Leu Lc60 and Leu Lc83 grew in MRS agar pour-plates containing tetracycline, while starter streptococci (strains 584 and 134) did not.

Morphology. Both isolates grown either in MRS broth or in RSM, appeared similar to type-strain leuconostocs when examined microscopically. Both cultures appeared as short oval rods, in pairs and short chains.

Lactic Acid Isomer. The isomer of lactic acid formed during growth in RSM culture was determined by measuring the concentrations of L- and D-lactate enzymatically. Both isolates produced significant quantities of D-lactate.

Temperature Optimum for Growth. The optimum temperature for growth of both strains was determined by determining the change in pH in a yeast-milk culture incubated in a temperature gradient incubator. The optimum temperature for Lc60 was 28.0 C, and for Lc83 was 28.5 C.

Growth on Different Sugars. The ability of the two isolates to grow on various sugars was determined by inoculating suitable dilutions of MRS-grown cells into soft MRS agar with the glucose replaced by alternative sugars. The results are shown in Table xxxix, along with the data obtained with type-strain cultures. Both isolates grew on lactose and its

two constituent sugars (glucose and galactose), and on maltose. In addition, Lc60 grew on ribose, arabinose and trehalose, while Lc83 grew on sucrose.

Table xxxix. Growth of various leuconostoc isolates in MRS agar, with different growth sugars.

Culture	Growth sugar[1]							
	Glu	Gal	Lac	Rib	Suc	Mal	Ara	Tre
20095 Leu. dextranicum	+[2]	+	+	-	-	+	-	+
20082 Leu. mesenteroides	+	+	+	+	+	+	+	+
20096 Leu. paramesenteroides	+	+	-	+	+	+	+	+
20053 Leu. cremoris	+	+	-	-	+	-	-	-
Lc 60	+	+	+	+	-	+	+	+
Lc 83	+	+	+	-	+	+	-	-

[1] Glu = glucose, Gal = galactose, Lac = lactose, Rib = ribose, Suc = sucrose, Mal = maltose, Ara = arabinose, Tre = trehalose
[2] + = growth, - = no growth

Growth in RSM. The growth of the type strain of Leu. cremoris and of the two isolates in RSM at 30 C is shown in Fig 21. The doubling times were Leu. cremoris NZRCC 20053, 1.89 h; Lc60 1.43 h; and Lc83 1.21 h. Both NZRCC 20053 and Lc83 grew to maxima in excess of $2*10^7$ cfu/mL and remained at their maxima for at least 48 h. Isolate Lc60 only attained a maximum of $4*10^6$ cfu/mL, and the count started to decrease after 24 h.

III.3.2 The Effect of Glucose and pH on Citrate Utilization by Leuconostoc Lc83. The disappearance of citrate from a buffered solution containing washed cells of Lc83 grown in MRS

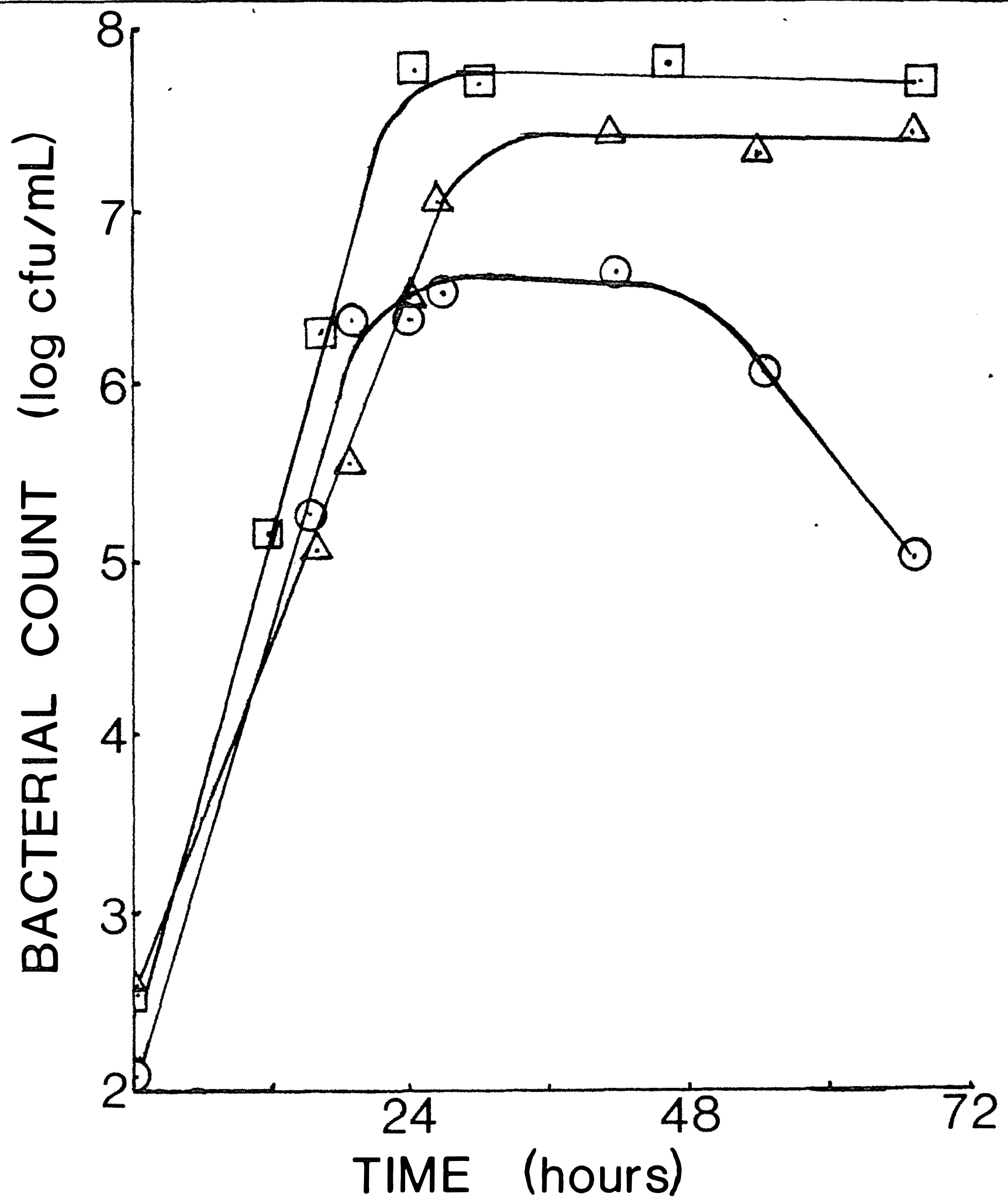


Figure 21. The rate of growth of three leuconostoc isolates in RSM at 30 C. One hundred mL volumes of sterile RSM at 30 C were inoculated with 1 mL of a 10^{-3} dilution of RSM grown leuconostoc isolates and cultured at 30 C. Cell counts were determined at various times by plating suitable dilutions into pour-plates of MRS agar and incubating at 30 C for 48 h. Leuconostoc cremoris NZRCC 20053, △-△; leuconostoc Lc 60, ⊙-⊙; leuconostoc Lc 83, ⊡-⊡.

broth, is shown in Fig. 22. At a pH of 6.25, little citrate disappeared in 1 h however the addition of 1mM glucose at intervals of 15 min caused detectable citrate disappearance. By contrast, at pH 5.25, half of the citrate had disappeared within 1 h in the absence of any sugar, and in the presence of glucose, citrate disappearance was rapid. No citrate remained after the second addition of glucose.

III.3.3 Growth and Citrate Utilization by Leuconostoc Isolates in Gouda-type Cheeses.

III.3.3.1 Statistical Approach. Twelve vats of cheese were manufactured on 6 days over a period of 8 weeks. On each manufacturing day, the curd in one vat was dry salted before pressing, and the resulting cheese were matured at either 10 or 15 C. The curd from the other vat was also pressed into two cheeses and one was brine salted. The second cheese remained unsalted throughout its life. These two cheeses were matured at 15 C.

Three leuconostoc cultures were added on different occasions to the cheese-milk with the acid-producing starter. The same culture was added to both vats on each occasion. On three occasions, Lc83 was used, Lc60 was used twice, and the type-strain of *Leu. cremoris* (strain 20053) was used once. The results presented below are the mean values of any replicates.

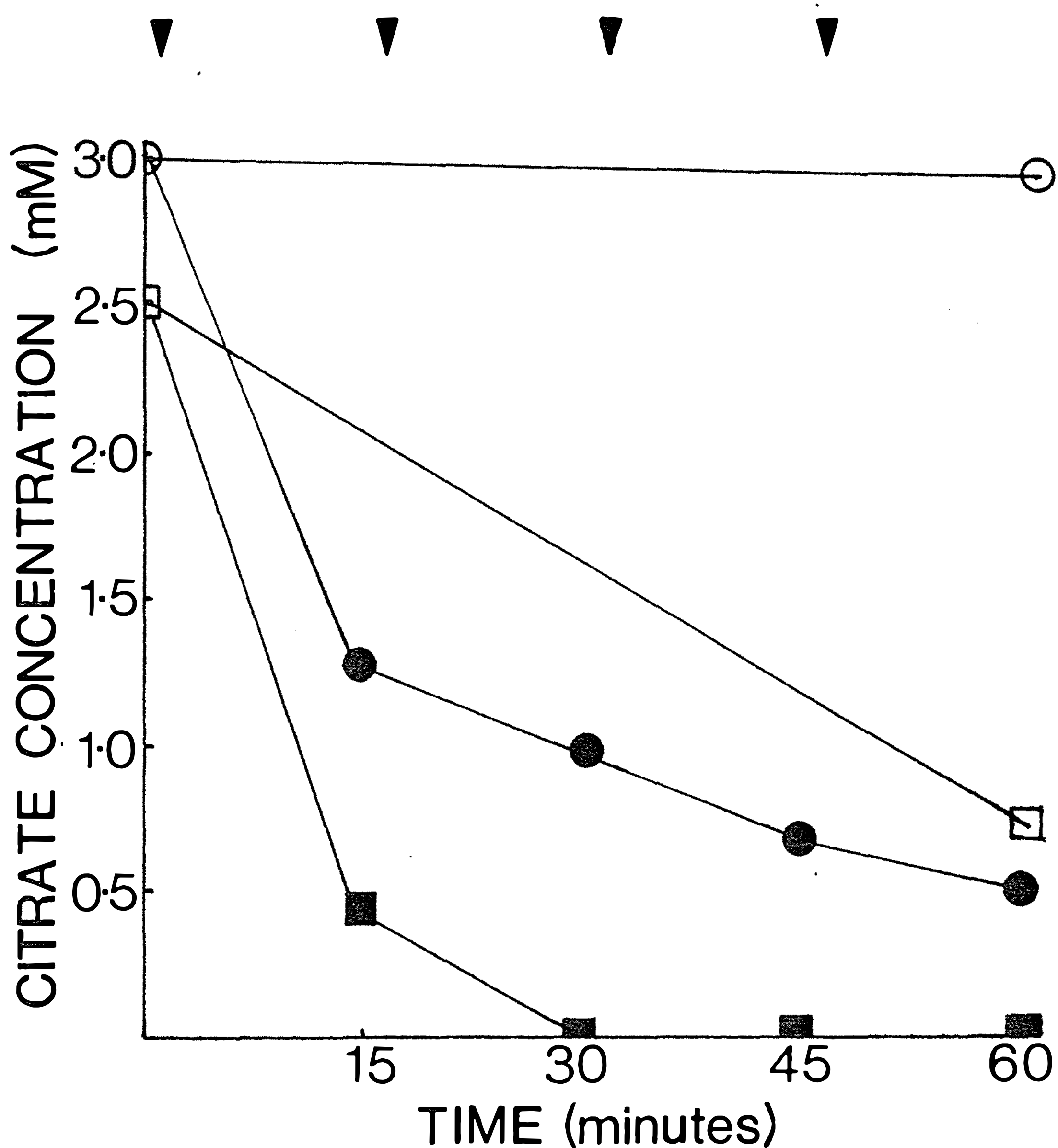


Figure 22. The effect of glucose and pH on the utilization of citrate by Lc83. Cells of leuconostoc Lc83 were grown in MRS broth, harvested by centrifugation and washed with 2 volumes of MES buffer (50mM). The washed cells were inoculated into MES buffer (50mM) at 30 C containing 3mM citrate, 50mM NaCl and 10mM $MgCl_2$ at either pH 6.25 (⊙-●) or 5.25 (⊡-■). Control flasks contained no sugar (open symbols) while 50 ul aliquots of glucose (final concentration 1mM) were added to experimental flasks at 15 min intervals, immediately after removal of samples for analysis. The concentration of citrate was determined enzymatically. Control, pH 6.26, ⊙-⊙; control 5.25, ⊡-⊡; experimental 6.25, ●-●; experimental 5.25, ■-■.

III.3.3.2 Cheese Composition. The mean results of the analysis of all 12 cheeses are presented in Table xc. The means for each factor are within the bounds of normally acceptable Gouda-type cheeses in N.Z. and the standard deviations show that the variations between cheeses are small.

Table xi. Composition of "Gouda" cheeses made in the aseptic vats.

	Mean	s.d.
Moisture (%)	40.8	1.0
Fat (%)	30.2	1.3
MNFS (%)	58.5	1.0
FDM (%)	51.0	1.7
SNF (%)	29.0	0.9
Ca (mM/kg)	195	9
Ca/SNF (mM/kg)	6.73	0.34
Salt-dry salted (%)	1.68	0.26
S/M -dry salted (%)	4.07	0.62
Salt-brine salted (%)	1.46	0.21
S/M -brine salted (%)	3.60	0.50
pH 1d -dry salted	5.36	0.14
pH 1d -brine salted	5.20	0.06
pH after 3m at 10 C	5.37	
pH after 3m at 15 C	5.43	
pH after 3m brine salted	5.45	
pH after 3m no salt	5.51	

It was noticeable that the pH of the dry-salted cheeses was 0.16 units higher than that of the unsalted cheeses at 1 day, probably due to the inhibition of acid production by the early addition of the salt. However the pH of these cheeses did not change significantly during maturing, while the pH of the brined or unsalted cheeses rose to exceed that of the dry-salted cheeses by 3 months.

III.3.3.3 Bacterial Counts in the Vat Milks and the Cheeses. The vat milks contained no coliforms or micrococcus organisms (absent in 10 mL), or NSLAB (absent in 5*10 mL samples). The mean value for the total count in the milk before starter addition was 6.7 cfu/mL. No NSLAB organisms were detected in the cheese at any sampling time.

The counts of leuconostocs are shown in Figure 23. The counts presented are the means of all cheese with each individual leuconostoc inoculum, since there was no detectable difference in count between the cheese with different salting treatments, or between the dry-salted cheese held at the two temperatures.

In the cheese inoculated with the type-strain of *Leu. cremoris* (20053), the counts increased by a factor of 10 between inoculation and the end of pressing, due to whey removal. The maximum count was $2*10^5$ cfu/g. After pressing, the count remained fairly stable, being $1.4*10^5$ cfu/g at 1 month and $7*10^4$ cfu/g at 3 months. By contrast, in the cheese inoculated with strain Lc60, the count increased to $5*10^5$ cfu/g by 1 day, indicating that the culture had managed between 2 and 3 cell division cycles, since whey removal would have increased the count to $1*10^5$ cfu/g. From 1 day onward, the count decreased to be $1*10^4$ cfu/g at 1 month, and $1*10^2$ cfu/g at three months.

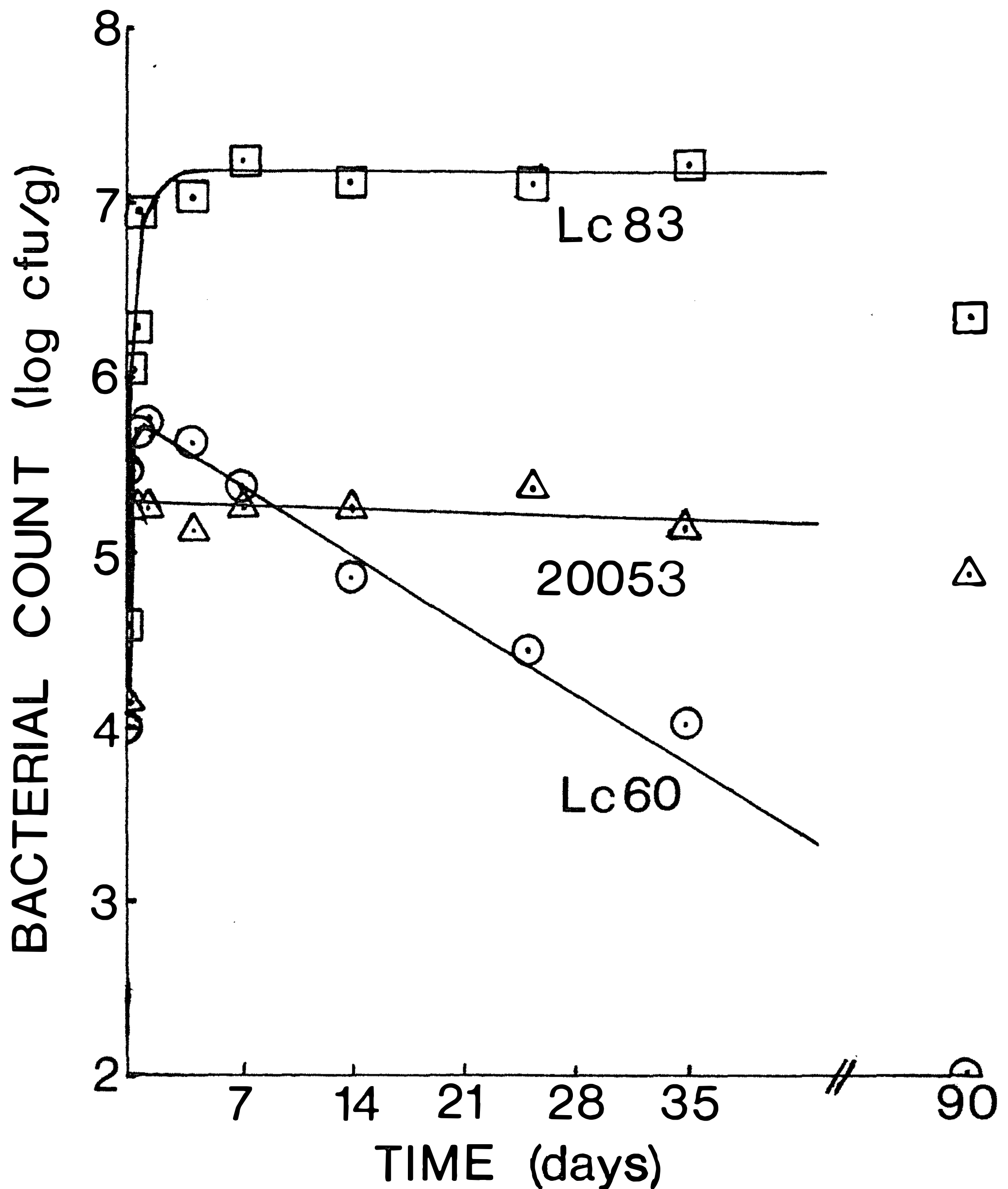


Figure 23. The counts of three leuconostoc strains inoculated into aseptically manufactured cheeses. Leuconostoc isolates were inoculated into aseptically manufactured washed-curd cheeses (Gouda-type). The counts of leuconostoc organisms in the cheeses at various ages was determined by plating samples in MRS agar containing tetracycline and incubating anaerobically at 30 C for 48 h. The data plotted are the means of all storage conditions and replicates for each leuconostoc organism, since no difference in count was observed whether the cheese was salted by addition of dry salt at hooping (with maturing at either 10 C or 15 C), was salted by brining (and matured at 15 C) or left unsalted (and matured at 15 C). Leuconostoc cremoris NZRCC 20053, △-△ (1 trial); leuconostoc Lc60, ⊙-⊙ (2 trials); leuconostoc Lc83, ⊡-⊡ (3 trials).

In cheese inoculated with culture Lc83, the count rose rapidly up to about day 4, reaching a maximum of $1.5*10^7$ cfu/g. This indicates at least 5 cell division cycles in the fresh cheese. The peak population was maintained throughout the first month, and then the count decreased to be $4*10^6$ cfu/g at three months.

III.3.3.4 Citrate Disappearance from Cheeses containing Leuconostocs. The concentrations of citrate in the cheeses with different leuconostoc inoculii are shown in Figure 24. In the cheese containing either strain 20053 or strain Lc60, there was a mean concentration of 0.149% citrate at 1 day, and no utilization in the first few weeks. The concentration in the cheese containing 20053 commenced to decrease after 25 days, but was still 0.09% after three months. In the cheese containing Lc60, a decrease was only noticed at three months.

In the cheeses containing Lc83 there was an initial reduction of 10% in the citrate concentration by day 1. Thereafter, the cheeses which were brine-salted or had no salt, showed a continuing decrease with no citrate being detectable by 3 months. In the cheeses with salt added before hooping, citrate disappearance was slower, the concentration at 3 months still being around 0.06%.

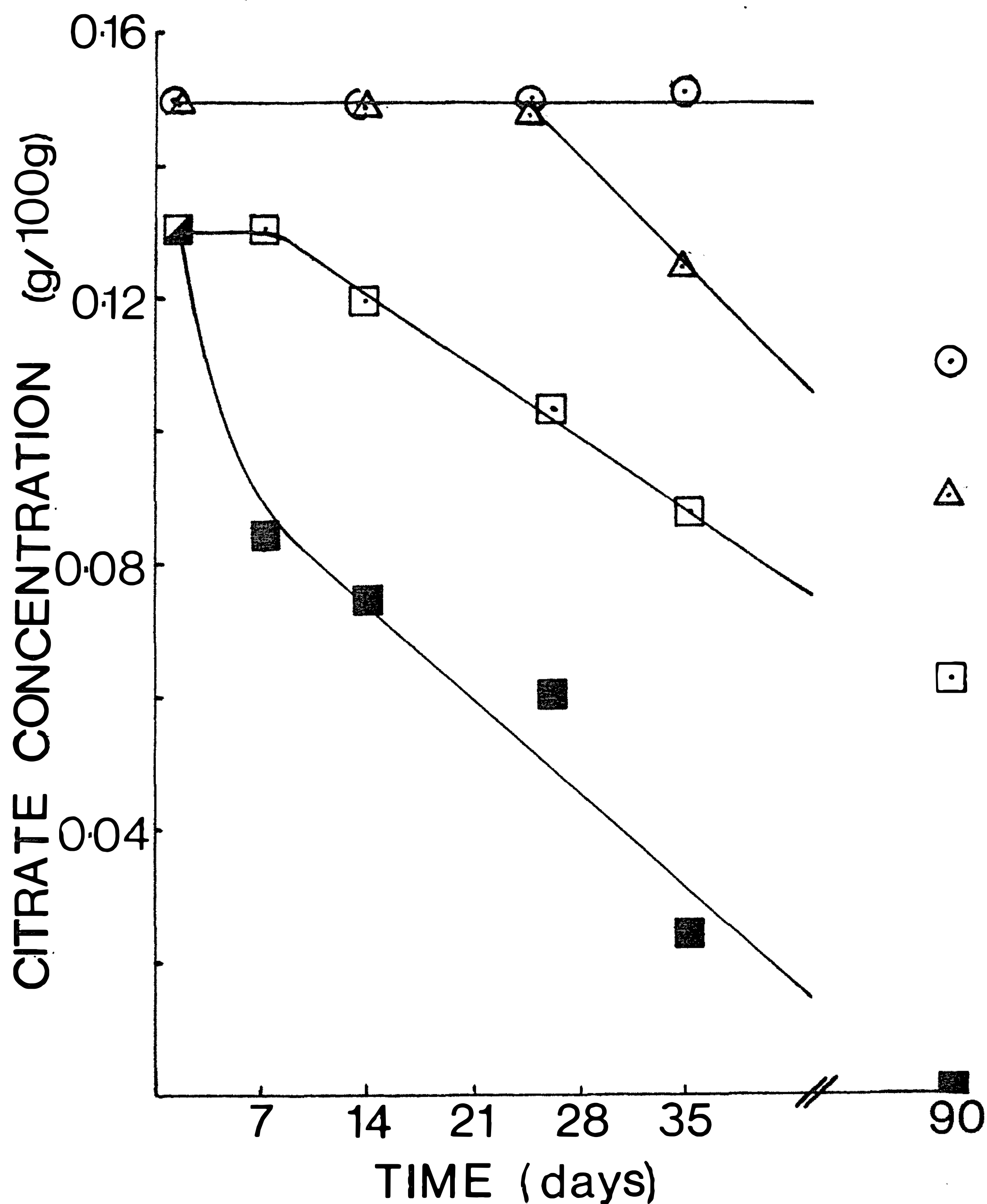


Figure 24. The disappearance of citrate from aseptically manufactured cheeses inoculated with one of three leuconostoc organisms. The concentration of citrate in aseptically manufactured washed-curd cheeses (Gouda-type) inoculated with various leuconostoc isolates was determined enzymatically during maturation up to 3 months. No difference was noted between the different storage conditions (see caption Fig 23) except for Lc83 where the data for dry-salted and brine or unsalted cheeses are shown separately. Leuconostoc cremoris NZRCC 20053, ▲-▲ (1 trial); leuconostoc Lc60, ⊙-⊙ (2 trials); leuconostoc Lc83 (dry-salted), ⊡-⊡ (3 trials); Leuconostoc Lc83 (both brine salted and unsalted), ■-■ (3 trials).

III.4 Discussion, Conclusions and Pointers for Further Research.

III.4.1 Strain Characterization. Both isolates were characteristic of leuconostocs in their resistance to tetracycline, their microscopic appearance and in the production of DL-lactate. The temperature optimum for growth indicated that both Lc60 and Lc83 were *Leu. cremoris* isolates, since, of the leuconostocs, only *Leu. cremoris* has a temperature optimum below 30 C (Garvie, 1974).

The sugar utilization patterns were more equivocal. In the current research, the type-strain of *Leu. cremoris* was galactose negative and sucrose positive, whereas it is reported to be galactose positive and sucrose negative (Riemelt, 1972, Garvie, 1974). The other type-strains tested reacted as published, except that the *Leu. dextranicum* culture did not grow on sucrose.

Neither Lc60 nor Lc83 exhibited a sugar utilization pattern which matched either the published patterns for *Leu. cremoris* or the type-strain pattern determined in this research. The Lc60 isolate matched most closely to the pattern of *Leu. paramesenteroides*, particularly since it grew on arabinose and trehalose, while the Lc83 isolate matched *Leu. cremoris* in all aspects except that it grew on maltose.

It is believed that the most appropriate leuconostoc in dairy fermentations is *Leu. cremoris* (hence its inclusion with *S. diacetylactis* as "aroma bacteria", Reiter and

Moller-Madsen, 1963; Waes, 1968). While Lc60 and Lc83 were not identical with the type-strain of Leu. cremoris in all respects, they bear the characteristics most necessary in cheese starters - the ability to grow on lactose and its constituent monosaccharides, and the ability to metabolize citrate.

III.4.2 Growth in RSM Culture. The three leuconostoc cultures tested were able to grow in RSM as rapidly as the NSLAB cultures reported in Section II.2. Furthermore, they were able to be grown consistently and reliably in RSM enabling this medium to be used to produce cultures for cheesemaking experiments. The generation times in RSM were around half those found by Goel and Marth (1969), however the maximum counts in RSM were approximately the same. Isolate Lc83 was as stable at the maximum count as was the strain CAF-16019 of Goel and Marth (1969), however Lc60 was not.

In unpublished experiments, the effect of growth stimulants noted by Goel and Marth (1971 a and b, and 1972) was confirmed, with a filter-sterilized extract of RSM-grown starter culture increasing the maximum population of both Lc60 and Lc83, but not increasing the generation time.

III.4.3 Citrate Utilization in Buffer. The utilization of citrate by Lc83 in buffer without sugar agrees with the findings of Mizuno and Jezeski (1959), that leuconostocs can utilize citrate in the absence of sugar, particularly at low

pH. The ability to utilize citrate in the absence of sugar should confer on a culture the ability to utilize citrate in washed-curd cheeses where the lactose is rapidly exhausted by curd-washing, and the initial lack of salt.

Citrate Utilization in Washed-Curd Cheeses. The aseptically manufactured cheeses had compositions very close to the target for Gouda-type cheeses in N.Z. The lack of NSLAB during maturation showed the efficacy of the aseptic vats and the surface waxing. Only Lc83 grew substantially after whey removal, and only in cheese with this culture was there a consistent utilization of citrate during maturation. Furthermore the cheeses with the lowest citrate utilization were the ones with Lc60, whose count rapidly decreased during maturation. It would appear that in order to ensure significant citrate utilization the leuconostoc culture must have the ability to grow during pressing, and to remain stable in the cheese for at least a number of weeks.

In dry-salted cheeses the rate of citrate utilization was not increased by maturation at 15 C. However, in the dry-salted cheeses citrate was utilized much more slowly than in the brine-salted or unsalted cheeses. Therefore, to ensure rapid citrate metabolism, it seems necessary to have low salt levels.

While the sugar content of the cheeses was not measured, it is highly likely since the curd was washed during manufacture, salted at low rates (or left unsalted), and held initially at

12 C, that there was no lactose present after 1 day. Thus the ability of Lc83 to utilize citrate in the absence of sugar would also contribute to its ability to utilize the citrate present in the cheese.

The lack of growth or citrate utilization by either Lc60 or NZRCC 20053, indicate that neither is an appropriate strain for use in cheese where significant citrate utilization is required. Since Lc60 has been used extensively for the production of Gouda-type cheese in N.Z., it must be questioned whether the eye-formation noted was a result of the addition of the leuconostoc.

III.4.4 Pointers to Further Research. Despite the volumes of published material on the benefits of addition of leuconostocs in the starter, little information is available on the exact characteristics required of the culture. The data reported in this section has established that for citrate utilization in the cheese, the leuconostoc strain should be able to grow and remain stable over an extended period, as shown for Lc83 in both milk and cheese. It must also be able to utilize citrate in the absence of lactose. Further research is required to establish a pool of leuconostoc cultures with known growth characteristics and to determine the metabolic products of lactose and citrate utilization in RSM and in cheese. It would then be possible to determine under what conditions leuconostocs are stimulated to utilize citrate, and what products are produced. If diacetyl flavour is desired, it may

be prudent to include an acetaldehyde producing starter, particularly S. thermophilus, in the starter since it has been shown (Collins and Speckman, 1974) that acetaldehyde promotes acetoin and diacetyl production by Leu. cremoris.

Since S. diacetylactis is incapable of metabolizing citrate in the absence of sugar, the leuconostoc component of an aroma-producing starter will be the only organism present in large numbers in the ripening cheese which is capable of increasing the aroma. A better understanding of the conditions which stimulate this metabolism will enable the cheesemaker to more reliably produce those cheeses which rely on the production of aroma compounds for their distinct flavours.

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